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Genetic Study of Three Porcine Mycoplasma Species and *Mycoplasma fermentans*

by

Yiping Huang ©

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Genetic Study of Three Porcine Mycoplasma Species and Mycoplasma Fermentans** submitted by Yiping Huang in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

I dedicate this thesis

to my parents

for their love and understanding over the years.

ABSTRACT

Mycoplasmas (class *Mollicutes*) are wall-less prokaryotes, among the smallest and simplest self-replicating organisms. Most mycoplasmal rRNA genes studied have a gene order similar to other prokaryotes, *i.e.* 5'-16S-23S-5S-3'. Moreover, the three genes are closely linked to each other and function as a transcription unit. However, unusual rRNA gene organizations have been found in several mycoplasmas, such as *Mycoplasma hyopneumoniae* and *Mycoplasma gallisepticum.* The rRNA gene organization could be used as a criterion to study the genetic relatedness among mycoplasma species.

In this thesis, the macro-restriction maps of the genomes from two closely related porcine mycoplasma species, *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*, have been constructed using field-inversion gel electrophoresis. It has been noted that *M. flocculare*, like *M. hyopneumoniae*, has a single set of rRNA genes (16S, 23S, and 5S) and an orphan 5S rRNA gene which is separated from the closely linked 16S-23S rRNA gene cluster by at least 150 kbp.

To study the transcription regulation of the orphan 5S rRNA gene, a 665 bp Xba I fragment containing this 5S rRNA gene was cloned from *M. flocculare* DNA. By comparing the sequence of the *M. flocculare* 5S rRNA gene and its flanking regions with that of the *M. hyopneumoniae* 5s rRNA gene, it was found that the sizes of the 5S rRNA genes from both organisms were 106 bp with a homology of 95.3%. However, the homology of the flanking regions is very low. In the -10 regions, both organisms have the sequence, TATAATA, which matches the highly conserved promoter sequence found in this region for other "housekeeping genes" of most other prokaryotes. A stem-loop terminator structure has been noted at the 3' end of the *M. flocculare* 5S rRNA gene. The data suggest that the *M. flocculare* 5S rRNA gene could be regulated by its own promoter and terminator.

The physical map of the genome of *M. fermentans* (*incognitus* strain), a human mycoplasma which has attracted considerable attention recently because of its frequent isolation from AIDS patients, was constructed using field-inversion gel electrophoresis. The location of the rRNA genes (16S, 23S, and 5S) was determined on this map. The *M. fermentans* (*incognitus* strain) 980 kbp genome contained two copies of each rRNA gene (16S, 23S, and 5S). The 16S and 23S rRNA genes are each closely linked as two 16S-23S rRNA gene clusters. Both 5S rRNA genes are separated from these gene clusters. Therefore, the three rRNA genes (16S, 23S, and 5S) cannot be co-transcribed.

The results from this study indicate that comparison of the rRNA gene organization can be useful to study evolution relationship among mycoplasma species.

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I would like to thank my supervisor Dr. G. W. Stemke for his advice, help, and patience through all these years. I owe a very special thanks to Dr. K. L. Roy for his constructive and valuable advice on my work. I would also like to offer my gratitude to the members of my supervisory committee: Drs. S. E. Jensen, J. A. Robertson, H. Anwar, for their helpful advice on academic matters. Critical examination of the thesis by Drs. M. Stiles and P.-C. Hu was appreciated.

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TABLE OF CONTENTS

Page

CHAPTER I. INTRODUCTION

1.1. Overview of the class *Mollicutes* 1

1.2. The relationships between mycoplasmas and bacteria 2

1.3. Porcine mycoplasmas 4

  1.3a. *Mycoplasma hyopneumoniae* 5

  1.3b. *Mycoplasma flocculare* and its relationship with *Mycoplasma hyopneumoniae* 6

  1.3c. *Mycoplasma hyorhinis* 7

1.4. *Mycoplasma fermentans* and its role in human respiratory disease and AIDS 9

1.5. Overview of the ribosomal RNA 11

1.6. rRNA genes in prokaryotes 12

1.7. Transcriptional regulation and processing of rRNA 14

1.8. Ribosomal RNA genes in *Mollicutes* 17

1.9. Application of restriction pattern analysis and hybridization pattern analysis to study genetic relatedness among mycoplasmas and the organization of mycoplasma rRNA genes 20

1.10. The model of the secondary structure of the 5S rRNA 23

1.11. The concept of field-inversion gel electrophoresis 25

1.12. The application of field-inversion gel electrophoresis in studying the genome of mollicutes 27

1.13. Research objectives 29

CHAPTER II. MATERIALS AND METHODS

2.1. Chemicals and enzymes 31

2.2. Bacterial Strains, plasmids and bacteriophages 35

2.3. Growth of mycoplasma 35

2.4. Growth of *E. coli* 36

2.5. Genomic DNA isolation 36

2.6. Total RNA purification 37

2.7. Primer extension analysis 37

2.8. Cloning of the *M. flocculare* 5S rRNA gene 38

2.9. Isolation of M13 recombinant single stranded and double stranded Rf DNA 39

2.10. DNA sequence analysis 40

2.11. Methodology of the computer analysis 41

2.12. Preparation of intact mycoplasma chromosomal DNA and restriction digestion 41

2.13. Preparation of the size marker 42

2.14. Field-inversion gel electrophoresis 42

2.15. Contour-clamped homogeneous electric field electrophoresis 42

2.16. Conventional electrophoresis 43

2.17. Double restriction endonuclease digestion 43

2.18. Southern analysis 44

2.19. Preparation of large DNA fragments probes 45

2.20. Random primer DNA labeling 45

2.21. Kinase 5' end labeling 46

2.22. Preparation of cloned *M. hyopneumoniae* genomic DNA probes 46

2.23. Preparation of the rRNA probes 46

CHAPTER III. RESULTS

3.1. Comparison of the DNA restriction cleavage patterns of *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare* 49

3.2. Comparison of the genomic DNA cleavage patterns of *M. hyorhinis* and other two porcine mycoplasma species 55

3.3. Hybridization of the rRNA probes with chromosomal DNAs from *M. flocculare* and *M. hyopneumoniae* 56

3.4. Hybridization of the rRNA probes with *M. hyorhinis* chromosomal DNAs 60

3.5. Sizes of the *M. hyopneumoniae*, *M. flocculare*, and *M. hyorhinis* genomes 61

3.6. Construction of macro-restriction maps of *M. hyopneumoniae* and *M. flocculare* genomes 65

3.7. Localization of the rRNA genes on the *M. hyopneumoniae* and *M. flocculare* genomes 70

3.8. Comparison of the DNA restriction cleavage patterns of the two "*M. fermentans*" strains 71

3.9. Hybridization of the rRNA probes with *M. fermentans* (*incognitus* strain) and Mfer-A chromosomal DNA 77

3.10. The genome sizes of "*M. fermentans*" strains 78

3.11. Construction of macro-restriction map of *M. fermentans* (*incognitus* strain) genome 82

3.12. Localization of the rRNA genes on the *M. fermentans* (*incognitus* strain) genome 88

3.13. Construction of a preliminary macrorestriction map of Mfer-B 90

3.14. Cloning of the *M. flocculare* 5S rRNA gene 93

3.15. Sequencing strategies 94

3.16. Sequence of the *M. flocculare* 5S rRNA gene 94

CHAPTER IV. DISCUSSION

4.1. Comparison of the restriction endonuclease cleavage patterns of the chromosomalDNAs from *M. hyopneumoniae* and *M. flocculare* and their rRNA gene hybridization patterns 99

4.2. Comparison of the restriction endonuclease patterns and the rRNA gene hybridization patterns of *M. hyorhinis* with other two porcine mycoplasmas 103

4.3. Physical maps of *M. hyopneumoniae* and *M. flocculare* and the localization of the rRNA genes on these maps 105

4.4. Comparison of the restriction patterns of "*Mycoplasma fermentans*" strain: Mfer-A and Mfer-B, and *incognitus* strain (Mi) 109

4.5. Physical map of *M. fermentans* (*incognitus* strain) and localization of the rRNA genes on the map 110

4.6. The preliminary restriction map of Mfer-B 111

4.7. A model of the secondary structure of the *M. flocculare* 5S rRNA 112

4.8. The putative transcription regulation mechanism of the *M. flocculare* 5S rRNA gene 117

REFERENCES 122

LIST OF TABLES

Table Page

2.1. Restriction enzymes used in this study 32

2.2. Primers used in this study 34

3.1. Digestion of the intact *M. hyopneumoniae*, *M. flocculare* and *M. hyorhinis* genomic DNA with restriction restriction enzymes 54

3.2. Hybridization data of restriction fragments from *M. hyopneumoniae*, *M. flocculare*, and *M. hyorhinis* probed with the *E. coli* 16S, 23S, and 5S rRNA probes 59

3.3. Length and numbering of restriction fragments of *M. flocculare* and *M. hyopneumoniae* chromosome 63

3.4. Length and numbering of restriction fragments of *M. hyorhinis* chromosome 64

3.5. Hybridization data of *M. flocculare* and *M. hyopneumoniae* DNA restriction fragments 66

3.6. Digestion of Mfer-A and Mfer-B genomic DNA and *M. fermentans* (*incognitus* strain) genomic DNA with restriction restriction enzymes 72

3.7. Length and numbering of restriction fragments of *M. fermentans* (*incognitus* strain) chromosome 80

3.8. Length and numbering of restriction fragments of the Mfer-A and Mfer-B chromosomes 81

3.9. Hybridization data of *M. fermentans* (*incognitus* strain) DNA restriction fragments 86

3.10. Hybridization data of Mfer-B DNA restriction fragments 91

4.1. The length of the 5S rRNA from *E. coli*, *B. subtilis*, and mycoplasma species 114

LIST OF FIGURES

Figure page

3.1. Conventional agarose gel electrophoresis of restriction endonuclease digests of *M. hyopneumoniae* and *M. flocculare* DNA 50

3.2. FIGE of restriction endonuclease digests of intact *M. flocculare* genomic DNAwith restriction endonucleases...................................... 52

3.3. FIGE of restriction digests of intact *M. hyopneumoniae* genomic DNA with restriction endonucleases 53

3.4. Southern analysis of restriction endonuclease digests of *M. hyopneumoniae* and *M. flocculare* DNA with the 5S rRNA probe 58

3.5. Macrorestriction maps of the (a) *M. flocculare* ATCC 27716 genome, and (b) *M. hyopneumoniae* ATCC 25095 genome 68

3.6. CHEF of restriction digests of the intact *M. fermentans* (*incognitus* strain) chromosome DNA with restriction endonucleases......................... 73

3.7. FIGE of restriction digests of the intact *M. fermentans* (*incognitus* strain) chromosome DNA with restriction endonucleases....................... 74

3.8. CHEF of restriction digests of the intact Mfer-A chromosome DNA with restriction endonucleases.. 75

3.9. FIGE of restriction digests of the intact Mfer-B chromosome DNA with restriction endonucleases.. 76

3.10. Southern analysis of FIGE of restriction endonuclease digests of intact *M. fermentans* (*incognitus* strain) genomic DNA with the *Mlu* I5 probe...... 84

3.11. Southern analysis of FIGE of restriction endonuclease digests of intact *M. fermentans* (*incognitus* strain) genomic DNA with the *Bgl* I1 probe 85

3.12. Macrorestriction map of the *M. fermentans* (*incognitus* strain) genome 87

3.12. Southern analysis of FIGE of restriction endonuclease digests of intact *M. fermentans* (*incognitus* strain) genomic DNA with the 5S rRNA probe........ 89

3.14. Macrorestriction map of the Mfer-B genome 92

3.15. Complete double-stranded nucleotide sequence of the cloned *M. flocculare Xba* I fragment 96

3.16. Nucleotide sequence of the *M. flocculare* 5S rRNA gene and the flanking regions and sequence alignment with that of the *M. hyopneumoniae* 5S rRNA gene 97

3.17. Putative terminator structure at the 3' end of the *M. flocculare* 5S rRNA gene 98

4.1. Comparison of the *M. flocculare* 5S rRNA with that of eubacteria and mycoplasma species 115

4.2. Model of the secondary structure of the *M. flocculare* 5S rRNA 116

4.3. Model of the secondary structure of the prokaryotic 120N type (Gram negative) 5S rRNA 118

4.4. Model of the secondary structure of the prokaryotic 116 N type (Gram positive) 5S rRNA 119

LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
AMV	Avian myeloblastosis virus
ATCC	American Type Culture Collection
bp	Base pair (s)
BSA	Bovine serum albumin
CHEF	Contour-clamped homogeneous electric fields electrophoresis
Ci	Curie
dA	2'-deoxyadenosine
dATP	2'-deoxyadenosine-5'-triphosphate
dC	2'-deoxycytidine
dCTP	2'-deoxycytidine-5'-triphosphate
dG	2'-deoxyguanosine
dGTP	2'-deoxyguanosine-5'-triphosphate
DNase	Deoxyribonuclease
dT	2'-deoxythymidine
DTT	Dithiothreitol
dTTP	2'-deoxythymidine-5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
FIGE	Field-inversion gel electrophoresis
kbp	1000 base pair(s)
kcal	1000 calorie
Mf	*M. flocculare*
Mfer-A	*M. fermentans* ATCC19989 culture of Oct. 5, 1985
Mfer-B	*M. fermentans* ATCC19989 culture of Apr. 4, 1992
Mhr	*M. hyorhinis*
Mhp	*M. hyopneumoniae*
Mi	*M. fermentans* (*incognitus* strain)
mRNA	Messenger RNA
PEG	Polyethylene glycol
RNase	Ribonuclease
rrn	rRNA operon
rRNA	Ribosomal RNA
SDS	Sodium dodecylsulfate
TBE	Tris-EDTA borate bufer
TE	Tris-EDTA buffer
TEA	Tris-EDTA acetate buffer
tRNA	Transfer RNA
UV	Ultraviolet

CHAPTER I. INTRODUCTION

1.1. Overview of the class *Mollicutes*.

Mollicutes, the prokaryotes lacking a true cell wall and bounded by a single bilayered membrane, are among the smallest and simplest free living self-replicating organisms. Their genome sizes range from 577-590 kbp for *Mycoplasma genitalium* to 1730 kbp for *Asteroleplasma anaerobium* (Bove *et al.*, 1990; Colman *et al.*, 1990; Neimark and Lange, 1990; Pyle *et al.*, 1988; Robertson *et al.*, 1990; Su and Baseman, 1990; Weisburg *et al.*, 1989). Most species recognized thus far can be grown on artificial cell-free media of diverse complexity. Colonies are minute and often have a characteristic " fried egg " appearance on solid media. Although many genera of *Mollicutes* have unique morphology, *Mollicutes* as a whole may be regarded as pleomorphic (Hayflick, 1971; Razin, 1978; Weisburg *et al.*, 1989). The circular double-stranded genome of *Mollicutes* is distinguished by its minute size and low guanine-plus-cytosine (G+C) content. The G+C content of *Mollicutes* ranges from 24 to 46 mol%, with most having approximately 33 mol% (Bove, 1984; Christiansen and Erno, 1982; Christiansen *et al.*, 1975; Razin *et al.*, 1983; Stemke *et al.*, 1985). *Mollicutes,* which belong to the division *Tenericutes* (Murray, 1984), have four orders. Order I: *Mycoplasmatales,* which has one family: *Mycoplasmataceae* (with two genera, *Mycoplasma* and *Ureaplasma*); order II: *Entomoplasmatales* (Tully *et al.*, 1993), which has two families: *Entomoplasmataceae* (with two genera, *Entomoplasma* and *Mesoplasma*), *Spiroplasmataceae* (with one genus *Spiroplasma*)(Tully, 1989); order III: *Acholeplasmatales* (Edward and Freundt, 1970), which has one family: *Acholeplasmataceae* (with one genus *Acholeplasma*); order IV: *Anaeroplasmatales* (Robinson and Freundt, 1987), which has one family:

Anaeroplasmataceae (with two genera, *Anaeroplasma* and *Asteropleplasma*). In this thesis, the term "mycoplasmas" will be used to denote any species in the class *Mollicutes*.

The genus *Mycoplasma* has the largest number of known species. Its major characteristic is a strict need for exogenous sterol (Edward, 1971; Razin and Freundt, 1984). Members of the genus *Ureaplasma* are similar to *Mycoplasma* species in sterol requirements, but they need exogenous urea for growth (Shepard *et al.*, 1974; Shepard and Masover, 1979; Razin and Freundt, 1984). The acholeplasmas and asteroleplasmas are able to grow in the absence of sterols (Edward and Freundt, 1970; Freundt and Edward, 1979; Tully, 1979, 1989), whereas spiroplasmas and anaeroplasmas require sterol (or serum) for growth. Anaeroplasmas and asteroleplasmas are strict anaerobes and are found only in the bovine or ovine rumen (Robinson and Freundt, 1987); the other mollicute genera are facultative anaerobes (Razin and Freundt, 1984). The spiroplasmas, about which our knowledge is rapidly expanding, are helical in shape and occur in plant and insect hosts (Bove, 1984; Skripal, 1974, 1983; Tully *et al.*, 1987). Non helical mycoplasma-like organisms (MLO) are associated with plants and invertebrates (Tully, 1989).

1.2. The relationships between mycoplasmas and bacteria.

Present day cells reflect at least three primary lines of decent (Fox *et al.*, 1980; Woese, 1987). One to the true bacteria or eubacteria and the other to the archaebacteria. The third line is the eukaryote lineage. No two lines are more closely related to each other than either is related to the third one. The eubacterial line contains most of the recognized bacterial species, including all of the mycoplasmas (Fox *et al.*, 1980; Maniloff, 1983; Woese, 1987).

The class, *Mollicutes,* given the many unusual properties of its members, has aroused considerable interest about its origin and phylogeny. Early attempts to subdivide the mycoplasmas, by using immunological approaches, DNA composition, and the like, proved to be taxonomically useful but gave little phylogenetic information (Neimark, 1970 ; Sjostrom and Kenny, 1983). On the other hand, RNA-DNA hybridization (Neimark, 1983), DNA/DNA hybridization (Goldfine, 1982), lipid composition (Goldfine, 1982), and 16S rRNA (Woese *et al.,* 1980) oligonucleotide catalogs revealed the phylogeny of this group in considerable detail. As a consequence, our understanding of mycoplasma phylogeny and evolution has advanced dramatically during the last decade.

Some biologists regarded the mycoplasmas as living relics of a primitive type of cell that preceded present-day bacteria in evolution (Morowitz and Wallace, 1973). This model was an effort to explain the genome size data and postulated that genome doublings account for the two fold increase, from the 460 to 1000 megadaltons mycoplasma genome and then to the 2000 to 3000 megadaltons eubacterial genome. However, recent data from pulse-field gel electrophoresis do not support this model (Bove *et al.*, 1990; Neimark and Lange, 1990; Pyle *et al.*, 1988). It has been found that the sizes of the mycoplasmal genomes range continuously from 577-590 kbp to 1700 kbp (Bove *et al.,* 1990; Neimark and Lange, 1990; Pyle *et al.*, 1988; Robertson *et al.*, 1990; Su and Baseman, 1990; Weisburg *et al.*, 1989). The genome size of some large mollicutes almost overlap with some small walled bacteria (Bove *et al.*, 1990; Nuijten *et al.,* 1990).

The alternative model is that mycoplasmas represent degenerate eubacterial forms. The mycoplasmas might represent an artifactual taxonomic grouping of wall-less prokaryotes derived from many different eubacterial branches, or they might represent different phylogenetic forms on one major branch of the eubacterial tree (Maniloff, 1983). Studies by Neimark (1983) and Neimark and London (1982) led to their conclusion that acholeplasmas might be related to streptococci based on RNA-DNA hybridization studies and immunological cross reactivity of various enzymes. The initial characterization of mycoplasma 16S rRNAs by oligonucleotide cataloging revealed that they are related to the gram-positive bacteria with low G+C DNA composition; the general phenotype of these bacteria is clostridial. Within that group, the mycoplasmas are closely related to the bacillus-lactobacillus cluster, but they are related even more to a particular small subgroup of clostridia represented by *Clostridium innocuum* and *Clostridium ramosum* (Woese *et al.*, 1980).

Hori *et al.*(1981) sequenced the 5S rRNA of *Mycoplasma capricolum* and confirmed the close relationship between this species and gram-positive eubacteria. A more extensive phylogenetic analysis of mycoplasmas by Rogers *et al.*(1985) was based upon the 5S rRNA sequences of *C. innocuum* and 10 mycoplasmas. In addition to affirming earlier conclusions regarding mycoplasma phylogeny, the study indicated that the initial divergence of mycoplasmas from their clostridial ancestors probably involved the *Acholeplasma* branch, with further divergence of this stem leading to the sterol-requiring, anaerobic *Anaeroplasma* branch and other mycoplasma branches. A more recent study by Weisburg *et al.*(1989), based upon the 16S rRNA sequences of more than 40 species of mycoplasmas and six of their specific "walled" relatives, confirmed, refined, and extended their earlier studies and provided the basis for a phylogenetically valid taxonomy of the group. Now it has been widely accepted that mycoplasmas represent different phylogenetic forms on one major branch of the eubacterial tree (Maniloff, 1992).

1.3. Porcine mycoplasmas.

Mycoplasmas play a major etiologic role in porcine pneumonia. Examination by culturing of pig lungs with porcine pneumonia seen in slaughter-houses yields several mycoplasma species, the most prevalent ones being *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis*. *M. hyopneumoniae* plays the primary etiologic role in porcine pneumonia (Mare and Switzer, 1965, Goodwin *et al.*, 1965) while the precise role of *M.*

hyorhinis has been more difficult to determine. Another porcine mycoplasma of interest is *Mycoplasma flocculare* which is not implicated directly in porcine mycoplasma pneumonia (Friis, 1973; Razin and Freundt, 1984) but is closely related to *M. hyopneumoniae* (Rose *et al.*, 1979; Stemke *et al.*, 1985). *Mycoplasma hyosynoviae* (Ross and Karmon, 1970), *Mycoplasma sualvi* (Gourlay *et al.*, 1978), *Acholeplasma granularum*, *Acholeplasma laidlawii*, *Asteroleplasma anearobium*, and several other mycoplasmas have also been reported to be isolated from swine (Whittlestone, 1979; Weisburg *et al.*, 1989). In 1988, a new mycoplasma species, *Mycoplasma hyopharyngis*, was isolated from nasal and pharyngeal mucosae of clinically normal swine (Erickson *et al.*, 1988).

1.3a. *Mycoplasma hyopneumoniae*:

M. hyopneumoniae was first isolated by Mare and Switzer (1965) from lung tissue of field pigs with enzootic pneumonia of swine (EPS) in the United States. The organisms are tiny cocci, 0.4 µm in diameter, or coccobacillary to short filamentous cells about 0.5-0.75 µm in diameter. Colonies are very small, up to about 0.5 mm in diameter after 7-10 days incubation on solid media. They are usually convex with a granular surface. The typical " fried egg " appearance of most mycoplasma colonies is not normally seen in this species. They are considered facultative anaerobes. However, cultures in Friis broth grew faster and to high titers in air than in 8% CO_2 and cultures in air grew better when shaken than when stationary, although growth on agar is better under aerobic conditions with supplements of about 5% CO_2 (Stemke and Robertson, 1990). *M. hyopneumoniae* is nutritionally very fastidious and has been isolated only in the broth specifically devised for this organism, although isolated and adapted strains have been propagated in other media (Goodwin and Whittlestone 1966, Goodwin *et al.*, 1965, 1967; Mare and Switzer, 1966a, b; Whittlestone, 1979).

M. hyopneumoniae is the most important primary cause of EPS. The natural disease can be reproduced experimentally (Goodwin *et al.*, 1965; Hannan *et al.*, 1984; Mare and Switzer, 1965). In pneumonic lungs from pigs the organisms are found in greatest numbers adjacent to the cilia and plasma membrane of epithelial cells lining the bronchioles and bronchi. Infection of porcine tracheal ring organ cultures in combination with monolayer cultures of fetal lung fibroblasts produces progressive epithelial ciliostasis, loss of parallel arrangements and gentle curvature of cilia exfoliation, and some sloughing of epithelial cells. The organism has been detected only in the porcine respiratory tract and associated structures, including the nasal cavity and tonsils (Amanfu *et al.*, 1984; Razin and Freundt, 1984; Switzer, 1972; Tajima and Yagihashi, 1982; Whittlestone, 1972). The growth rate of infected pigs has been reduced by 12.7-15.9% (Pointon *et al.*, 1984). Studies by Williams and Gallagher (1978) and Geary and Walczak (1983) showed that whole cells and purified membranes of *M. hyopneumoniae* had cytopathic effects in porcine tracheal ring, porcine lung explant organ, porcine lung fibroblasts and MRC-5 human lung fibroblasts.

The DNA of *M. hyopneumoniae* has a G+C content of 27 to 33 mol% (Stemke *et al.*, 1985; Kirchhoff and Flossdorf, 1987). The genome size of the organism was estimated to be 1070 kbp by pulsed-field electrophoresis (Robertson *et al.*, 1990).

1.3b. *Mycoplasma flocculare* and its relationship with *Mycoplasma hyopneumoniae.*

M. flocculare was first isolated by Friis in Denmark (Meyling and Friis, 1972). *M. flocculare* is similar to *M. hyopneumoniae* in its fastidiousness, slow acid production, reluctance to colonize on agar, cellular morphology, and colonial morphology (Friis, 1972; Meyling and Friis, 1972; Whittlestone, 1979). *M. flocculare* shares many biological property with *M. hyopneumoniae*, *e.g.* sterol requirement, no arginine hydrolysis, no phosphatase production (Friis, 1972; Rose *et al.*, 1979; Stemke and Robertson, 1990). The polyacrylamide gel electrophoresis (PAGE) pattern of the cell proteins of the type strain of the species shows some similarities to that of the type strain of *M. hyopneumoniae*, but there are distinctive bands (Rose *et al.*, 1979; Razin and Freundt, 1984). Serological cross-reaction has been observed with ordinary serological methods (Friis, 1977; Friis and Jensen, 1984), enzyme-linked immunosorbent assay (Freeman *et al.*, 1984), and crossed immunoelectrophoresis (Ro and Ross, 1983). Several species-specific antigens were found in *M. hyopneumoniae*, but lacking in *M. flocculare* (Bolske *et al.*, 1987). The extent of DNA/DNA hybridization between *M. hyopneumoniae* and *M. flocculare* is about 10% (Stemke *et al.*, 1985).

Experimental infection of piglets by exposure to aerosols with low passage cultures of *M. flocculare* results in the development of small pneumonic lesions with peribronchial and perivascular accumulations of mononuclear cells, slight mononuclear and polymorphonuclear cell accumulations and damage to the surface epithelium of the nasal cavity. The brain, pleural and pericardial cavities are infected on rare occasions (Friis, 1973; Razin and Freundt, 1984). The organism has been isolated from the nasal cavity and pneumonic lungs of swine without EPS in Denmark and the United Kingdom (Whittlestone, 1979).

The mol% G+C content of the DNA is the same as *M. hyopneumoniae*, *i.e.* 27-33 mol% (Kirchhof and Flossdorf, 1987; Stemke *et al.*, 1985). The genome size of *M. flocculare* is about 890 kbp (Robertson *et al.*, 1990).

1.3c. *Mycoplasma hyorhinis.*

M. hyorhinis is a common inhabitant of the nasal cavity of both healthy and diseased pigs. It is one of major mycoplasmal contaminants of cell cultures (Leach and Butler, 1966; Whittlestone, 1979). The organism was first isolated by Switzer in 1950s (1955).

Many strains of this species are easy to grow, even on primary culture in a range of mycoplasma broths (Switzer, 1955; Whittlestone, 1979). However, it has been reported that the majority of *M. hyorhinis* strains isolated from tissue cultures cannot grow in normal mycoplasma media. Some of them can only grow in specially designed medium (Del Giudice and Hopps, 1978, Del Giudice *et al.*, 1980; Hay *et al.*, 1989). The mol% G+C of the *M. hyorhinis* genomic DNA is 27.3-27.8 %mol (Neimark, 1970; Stanbridge and Reff, 1979). The genome size of this organism is about 820 kbp (Weisburg *et al.*, 1989).

The organism is a common secondary invader in preexisting pneumonia of pigs primarily caused by *M. hyopneumoniae*. There is some indication that *M. hyorhinis* alone may be the cause of some cases of pneumonia occurring under natural conditions (Gois *et al.*, 1968; Whittlestone, 1979; Razin and Freundt, 1984). The organism is associated with at least some cases of porcine arthritis and polyserositis (Duncan and Ross, 1973, Ennis *et al.*, 1971; Ennis and Decker 1972).

Taylor *et al.* (1988) reported that genomic restriction fragments isolated from *M. hyorhinis* and *M. hyopneumoniae* were shown by DNA hybridization and nucleotide sequence analyses to contain sequences common to these two species, as well as to *M. flocculare*. Intraspecies hybridization experiments using these fragments as probes indicated that the sequence is highly redundant in several strains of *M. hyorhinis* . In contrast, repetition of the sequence was limited in *M. hyopneumoniae* and *M. flocculare*, and no homologies to this repeated element were apparent in mycoplasma species isolated from any animal host other than swine. *M. hyorhinis* was shown to undergo high-frequency phase transitions in colony morphology and opacity and in the expression of diverse lipid-modified, cell-surface protein antigens (Rosengarten and Wise, 1990, 1991).

1.4. *Mycoplasma fermentans* and its role in human respiratory disease and AIDS.

M. fermentans was first isolated by Ruiter and Wentholt (1953) from two patients with genital infections. Recently a new strain of this mycoplasma species, *Mycoplasma fermentans* (*incognitus* strain) was detected in spleen and Kaposi's sarcoma tissues from two AIDS patients (Lo, 1986; Lo *et al.*, 1989b, 1989c) as well as in several different tissues from patients with respiratory disease (Lo *et al.*, 1989a).

Mycoplasma fermentans has the relatively unusual biochemical properties of utilizing glucose both aerobically and anaerobically as well as having the ability to metabolize arginine. It is fastidious in cultivation requirements and has a small spherical particle size and occasional filamentous morphology. It forms only irregular and very small colonies with diffuse edges on agar plates (Razin and Freundt, 1984; Ruiter and Wentholt, 1953). The mol% G+C of the DNA is 27.5 - 28.7. The genome size of *M. fermentans* GII has been reported to be about 1160 kbp (Neimark and Lange, 1990). The new strain is even more fastidious than the type strain and fails to grow in most mycoplasma media other than modified SP-4 medium (Lo *et al.*, 1989b).

Mycoplasma fermentans has been isolated from patients with balanitis and vulvovaginitis, from the uterine tube of a patient with subacute salpingitis, from the synovia of patients with rheumatoid and nonrheumatoid arthritis, from bone marrow of leukemic and nonleukemic patients (Murphy *et al.*, 1970; Razin and Freundt, 1984; Ruiter and Wentholt, 1953). Inoculation of the organism directly into the uterine tubes of grivet monkeys, or into the uterine cavity followed by curettage of the endometrium, produced a self-limiting acute salpingitis and parametritis (Moller *et al.*, 1980). Intraperitoneal inoculation of large quantities of *M. fermentans* cells or washed membranes is lethal to mice and produces symptoms resembling the endotoxic effect of Gram-negative bacteria (Gabridge, 1974; Gabridge *et al.*, 1972). A cytotoxic effect is exerted on cultured mouse thymocytes (Gabridge and Schneider, 1975; Razin and Freundt, 1984). It has been suggested that this cytotoxic effect of *M. fermentans* might play some role in AIDS (Lo *et al.*, 1989c). The organisms induced leukemoid disease with lymphadenopathy in experimentally infected animals (Plata *et al.*, 1973). However, whether *M. fermentans* is truly pathogenic in humans had not been further investigated until recently.

In the late 1980s, a new strain (Strain "*incognitus*") was found in spleen, liver, lymph node, Kaposi's sarcoma tissues, or peripheral blood mononuclear cells from AIDS patients (Lo, 1986; Lo *et al.*, 1989c). It was also detected in the systematic necrotizing lesions in some previously healthy non-AIDS patients with an acute fatal disease (Lo *et al.*, 1989a). Experimental inoculation of the strain into silvered leaf monkeys produced fatal infections and the organism could be directly detected in necropsy tissues of these monkeys (Lo *et al.*, 1989d).

The finding of a high prevalence of *M. fermentans* infection in patients infected with human immunodeficiency virus type 1 (HIV-1) has raised the possibility that the two infectious agents may interact synergistically and that mycoplasmas could play a significant disease-promoting role in HIV-infected patients (Lo, 1992). Montagnier and associates (Lemaitre *et al.*, 1990) reported that the cell killing effect of HIV-1 in $CD4^+$ human lymphocytic CEM cell cultures could be significantly reduced by tetracycline antibiotics. The tetracycline-treated cultures continued to produce a high titer of HIV-1 in the absence of any cytocidal effect. In additional studies, mycoplasma was confirmed as the hidden tetracycline-sensitive prokaryotic agent in the cultures (Lemaitre *et al.*, 1992). The contaminating mycoplasma was later isolated from CEM U13 cells and identified as a strain of *M. fermentans* (Lemaitre *et al.*, 1992). It has been reported that coinfection with the *incognitus* strain significantly enhanced the ability of HIV-1 to induce cytopathic effects on human T-lymphocytes *in vitro* (Lo *et al.*, 1991; Lemaitre *et al.*, 1992).

1.5. Overview of the Ribosomal RNA.

Ribosomes are ribonucleoprotein particles that are responsible for the translation of the genetic code. Each ribosome is constructed of two subunits. In prokaryotes, the sedimentation constant of the smaller ribosome subunit is 30S (Svedberg units) and of the larger subunit is 50S; the intact ribosome is 70S. In eukaryotes, the two subunits are 40S and 60S; and the intact ribosome is 80S. The 30S subunit of the prokaryotic ribosome contains 21 proteins and a 16S RNA molecule while the 50S subunit contains 34 proteins and two RNA molecules, a 23S species and a 5S species. In eukaryotic cells the 40S subunit contain 30 proteins and a 18S RNA and the 60S subunit contain about 50 proteins and three RNA molecules (28S, 5.8S, 5S). Ribosomes typically contain 50 to 60 percent RNA as an integral part of their structure (Manor and Haselkorn, 1967; Nomura, 1970; Spirin, 1964, 1990).

Ribosomal RNA (rRNA) is one of the most highly conserved classes of macromolecules found in nature (Gutell *et al.*, 1985). This is a consequence of its central importance in protein synthesis and the fact that the fundamental mechanism of translation is based on interactions between rRNA, mRNA, and tRNA. In prokaryotic cells (in both the eubacterial and archaebacterial lines), ribosomes contain three ribosomal RNA molecules, usually called 5S, 16S, and 23S rRNA. These molecules contain about 120, 1540, and 2900 nucleotides, respectively (Brosius *et al.*, 1978, 1980; Browlee *et al.*, 1968; Noller, 1984). In eukaryotic cytoplasmic ribosomes, the three main classes of rRNA are usually called 5S (about 120 nucleotides), 18S or 17S (about 2000 nucleotides), and 28S or 26S rRNA (about 4000-5000 nucleotides). Most eukaryotic cytoplasmic ribosomes also contain a 5.8S rRNA, which corresponds to the 160 nucleotides at the 5'-terminus of the prokaryotic 23S rRNA (Brosius *et al.*, 1980; Jacq, 1981; Nazar, 1980; Walker and Pace, 1983; Noller, 1984); some insects also have a 2s rRNA corresponding to the 3'-25 nucleotides of the 5.8S rRNA (Jordan *et al.*, 1980; Noller, 1984).

1.6. rRNA genes in prokaryotes.

During the assembly of functional ribosomes, rRNA undergoes a variety of metabolic steps. Because the level of their expression is universally high and their function is indispensable, rRNA genes constitute a reliable model system for the study of organization and expression of organelle genomes throughout phylogeny (Srivastava and Schlessinger, 1990). In prokaryotic cells, the numbers of the rRNA transcription units (operons) on the genome vary among different species. No direct evidence relates the number of rRNA (rrn) operon copies in an organism to growth rate regulation, but slowly growing organisms tend to have fewer copies (Noller, 1984).

The *Escherichia coli* genome, which has been studied in great detail, contains seven rRNA operons (rrnA, B, C, D, E, G, and H), each at a different location (King *et al.*, 1986; Kiss *et al.*, 1977; Nomura *et al.*, 1984). Each operon has two promoters, P1 and P2, about 120 bp apart. The 16S rRNA gene is located about 190 nucleotides downstream of promoter P2, followed by a spacer region containing genes for tRNA, then by the 23S rRNA, and then by another spacer region with a 5S rRNA gene (Brosius *et al.*, 1981; de Boer *et al.*, 1979; Young and Steitz, 1979). Two terminator signals (hairpin structure) follow, one is adjacent to the 3' terminus of the 5S rRNA gene, the other is about 170 nucleotides downstream. In every *E. coli* rrn operon, 16S and 23S rRNA are each present in one copy, but the numbers of tRNA and 5S rRNA genes vary (Brosius *et al.*, 1981; Lund and Dahlberg, 1977; Lund *et al.*, 1976; Morgan *et al.*, 1977, 1980; Srivastava and Schlessinger, 1990).

Bacillus subtilis contains 10 rrn operons (La Fauci *et al.*, 1986), and only two of these operons contain tRNA in the spacer between 16S and 23S rRNA (Loughney *et al.*, 1982). The striking difference between *B. subtilis* and *E. coli* rrn operon organization is that *B. subtilis* has tandem ribosomal gene sets while in *E. coli*, the rrn operons are dispersed (Srivastava and Schlessinger, 1990).

The operon organization is similar in most other eubacteria, *i.e.* an order of 5'-16S-23S-5S-3'. The number of rRNA operons varies in different species of eubacteria. However, an unusual organization has been found in *Leptospira interrogans*, in which there are two genes for the 16S and 23S rRNA but only one gene for 5S rRNA on the chromosome. In addition the three rRNA genes are far apart instead of forming a cluster (Fukunaga and Mifuchi, 1989).

In archaebacteria, the number of rRNA operons varies from one to four in different species. In most archaebacteria species, in contrast to the two tandem promoters of *E. coli*, expression is driven by multiple tandemly arranged promoters (Dennis, 1985; Mankin and Kagramanova, 1988), which presumably operate with different efficiencies.

A very different organization is seen in sulfur-dependent thermoacidophiles (like *Desulfurococcus mobilis*), in which only one set of rRNA genes was found and the 5S rRNA gene is unlinked to the 16S and 23S rRNA genes and the 5S rRNA gene is then separately transcribed (Kjems and Garret, 1987), a situation somewhat similar to the archaebacteria *Thermoproteus tenax* (Kjems *et al.*, 1987). An intravening sequence was found within the 23S rRNA gene of *Desulfurococcus mobilis* as well (Kjems and Garret, 1985).

Another unusual feature is seen in the thermophile *Thermus thermophilus*. It has two copies of the rRNA genes, but the 16S rRNA gene is separated by at least 7.8 or 6.4 kbp from the 5' end of the 23S-5S rRNA sequences (Hartmann *et al.*, 1987), and is transcribed as an isolated transcription unit while the other transcription unit contains a 23S rRNA gene, a 5S rRNA gene, and a tRNA^{Gly} gene (Hartmann and Erdmann, 1989).

Recently it has been reported that there is a single gene for the 16S rRNA and two copies of the 23S and 5S rRNA genes in the lyme disease spirochete, *Borrelia burgdorferi*. The 23S and 5S rRNA genes are tandemly duplicated in the order 23S-5S-23S-5S and are apparently not linked to the 16S rRNA gene, which is situated over 2 kbp upstream from the 23S-5S duplication (Schwartz *et al.*, 1992).

In most bacteria, the complex rrn region, regardless of the details of organization, is transcribed into long precursor molecules that include 16S, 23S, 5S rRNA sequences and all spacer elements. In *E. coli,* the spacer regions flanking the mature RNA sequences are also highly conserved in various operons (Woese, 1987). Precursor sequences at the 5' end and 3' end of 16S and 23S rRNA contain complementary sequence tracts that form strong base-paired stems enclosing the sequence of the mature species (Young and Steitz, 1978; Bram *et al.*, 1980).

The conservation of features of the operon and its transcripts thus extends to most eubacteria studied thus far. In particular, cotranscription of 16S and 23S rRNA is very frequent, the long transcripts contain spacer tRNA and the sequences that enclose the mature 16S and 23S rRNA species in double-stranded stems. Not surprisingly, these features tend to be involved in the regulation of transcription and processing, and thus of ribosomal formation (Srivastava and Schlessinger, 1990).

1.7. Transcriptional regulation and processing of rRNA.

The synthesis of ribosomes in *E. coli* and other prokaryotes is finely tuned to the cell's exterior environment. In a constant environment (steady-state growth), the rate of ribosome synthesis is proportional to the square of the growth rate. Under these conditions the synthesis of the individual rRNA and ribosomal proteins (r-proteins) is coordinately controlled. Except at very low growth rates, there is no significant turn-over of ribosomal components or buildup of pools of free components (Gallant, 1979; Gausing, 1980; Lindahl and Zengel, 1986). Transcription of rRNA operons in *E. coli* (Cashel and Rudd, 1987) as well as in *M. capricolum* (Glaser *et al.*, 1981) is under negative stringent control.

RNA synthesis is regulated by a variety of specific signals encoded in the DNA, some of which specify start-sites (*i. e.* promoters) and others stop-sites (*i. e.* terminators) for the RNA polymerase. Transcriptional regulation is achieved by modulating the efficiency with which the polymerase can recognize and interact with (initiate or terminate transcription at) these sites (Rosenberg and Court, 1979).

The particular base sequence of the DNA with which the polymerase must interact in large part determines the overall effectiveness of the promoter or terminator signal. Moreover, a number of effector molecules are known which can positively or negatively influence this interaction between polymerase and DNA. In many cases, these effector molecules also recognize nucleotide sequence information in the DNA template which is adjacent to and/or overlaps the site of polymerase action. One would expect that all promoter sites, and likewise, all terminator sites, should contain certain structural commonalities required for the basic function of RNA polymerase at the site (Rosenberg and Court, 1979).

It was noted by Pribnow (1975a, b) and Schaller *et al.* (1975), after comparing several promoter sequences from bacteriophage T7 and fd, that they contained a seven bp sequence, generally homologous to the sequence TATAATG, which was centered about 10 bp upstream of the mRNA startpoint. The similar sequence has been found in promoters from many other prokaryotic genes. As judged by sequence homology alone, the conserved -10 region clearly extends for some 13 nucleotides and the extent to which particular positions within the region are conserved appears to vary widely (Rosenberg and Court, 1979; Lindahl and Zengel, 1986; Glaser *et al.*, 1992).

Sequence similarity has also been noted among promoters in a region about 35 bp preceding the mRNA startpoint (called the -35 region) (Takanami *et al.*, 1976; Seeberg *et al.*, 1977). This TTGACA hexanucleotide occurs within a larger region and exhibits weaker homology compared with the -10 region (Rosenberg and Court, 1979; Lindahl and Zengel, 1986; Glaser *et al.*, 1992).

Terminators in bacterial and phage DNA falls into two groups: factor-independent terminators and factor-dependent terminators. Factor-independent terminators are found at the end of many bacterial and phage operons. They generally include a GC-rich inverted repeat sequence and consecutive runs of d(AT) on either side of the GC-rich dyad symmetry. The RNA stem-and-loop structure causes RNA polymerase to pause on the template. Enzyme release occurs within the downstream AT-rich sequence. Factor-dependent terminators do not appear to exhibit any consistent set of structural homologies. Some of these terminators clearly retain the stem-and-loop structure which exhibit less stability than their independent terminator counterparts (*i.e.* primary A-U pairing rather than G-C pairing). To date, only two ancillary protein factors that participate with RNA polymerase in transcription termination have been characterized. These are Rho and NusA. One working theory is that Rho acts by first binding to nascent transcripts rather than DNA or polymerase, then moves along the RNA (possibly involving Rho NTPase activity) until it finds a paused RNA polymerase. Polymerase can be shown to pause at or near the substantial stem-and-loop structure of the terminator. Moreover, it was shown that mutations that destabilized the hairpin concomitantly eliminated pausing (Brosius *et al.*, 1981; Cheng *et al.*, 1991; Holmes *et al.*, 1983; Rosenberg and Court, 1979).

In most of the eubacteria studied to date, the complex rrn regions, regardless of the details of organization, are transcribed into long precursor molecules that include all of the spacer elements. The precursors will then be processed through multistep processing to produce mature rRNA: (a) primary processing leads to formation of intermediate precursors of rRNA from the long transcripts; and (b) secondary maturation steps produce mature termini of rRNA from precursors. For the *E. coli* rrn operons, the sequences flanking the mature 16S, 23S, and 5S rRNA have extensive complementarity. The long stem structures generated by pairing these sequences are the substrates for RNAase III, which performs the initial cut in processing the mature RNA (Young and Steitz, 1978; Bram *et al.*, 1980, Brosius *et al.*, 1981). In wild-type cells, processing is rapid and most rRNA is mature. Only 1-2% of rRNA is present as long precursors (King and Schlessinger, 1983). The primary step, RNAse III cleavage separating pre-16S, pre-23S , pre-5S rRNA, and pre-tRNA species, begins even before transcription of the rrn operon is complete. The secondary processing events, which depend on the preformation of the complex of rRNA and r-proteins, then produce the mature rRNA.

1.8. Ribosomal RNA genes in mycoplasmas.

Mycoplasmal ribosomes resemble typical eubacterial ribosomes in having a sedimentation coefficient of about 70S, three rRNA species (23S, 16S, and 5S), and about 50 protein species. 2D-PAGE analysis by Kawauchi *et al.* (1982) revealed 30 protein spots in the 50S subunits and 21 protein spots in 30S subunits of *Mycoplasma capricolum* ribosomes, corresponding to the number of proteins in eubacterial ribosomes. Moreover, *Mycoplasma capricolum* ribosomal proteins resembled those of eubacteria in size and electrophoretic mobility, the resemblance being most pronounced with ribosomal protein profiles of Gram-positive bacilli. An interesting, although somewhat expected, conclusion is that the genes for ribosomal protein genes are well conserved in the mycoplasma genome despite its smaller genetic capacity. Thus, a significant portion of the *M. capricolum* genome is dedicated to ribosomal synthesis (about 55 genes of an estimated total of 400 to 600 genes).

The best-characterized mycoplasmal genes are those encoding for rRNA. The surveys indicate that the genome of most mollicutes carries only one or two copies of rRNA genes compared to 7-10 copies in other prokaryotes (Amikam *et al.*, 1982, 1984; La Fauci *et al.*, 1986; Nomura *et al.*, 1984). The large number of rRNA operons in the eubacteria *E. coli* and *B. subtilis* might serve to amplify rRNA synthesis and facilitate extremely rapid cell growth and reproduction, a feature not shared by most mollicutes. The low number of rRNA genes in mollicutes is in line with the concept of economy in genetic information in microorganisms with a small genome. However, there seems to be no strict correlation between genome size and the number of rRNA genes. Thus, the *M. arginini* genome contains two copies of the rRNA genes, while *Spiroplasma citri*, with a genome of twice the size, contains only one copy of the genes (Amikam *et al.*, 1982, 1984).

The study of the structure and function of elements controlling the expression of rRNA operons in mycoplasmas has a special appeal. The small copy number of these operons in mycoplasmas is expected to provide a simpler system for study than that provided by bacteria having a multiplicity of rRNA operons. In addition, most of the mycoplasma rRNA genes studied thus far have a topology similar to other prokaryotes, that is the classical order of 5'-16S-23S-5S-3'. Moreover, the three genes are close to each other and function as a single transcription unit (Amikam *et al.*, 1982, 1984; Frydenberg and Christiansen, 1985; Glaser *et al.*, 1984, 1992; Gobel *et al.*, 1984; Harasawa *et al.*, 1992; Iwami *et al.*, 1984; Neimark, 1983; Razin, 1985; Razin *et al.*, 1983a, 1984b; Sawada *et al.*, 1981, 1984). For most mycoplasma, no spacer tRNA genes have been found between 16S rRNA gene and 23S rRNA gene. However, the rRNA operons of the few MLOs examined so far carry a single tRNA^{Ile} gene in the spacer region between the 16S and 23S rRNA genes (Kirkpatrick *et al.*, 1990, Lim and Sears, 1989). *Acholeplasma laidlawii* also has tRNA genes in the 16S-23S spacer of the rRNA operon (Nakagawa *et al.* 1992). Prokaryotic rRNA probes can be used in cloning and mapping of mycoplasma rRNA genes (Amikam *et al.*, 1984; Razin, 1985).

M. capricolum was shown to carry two rRNA operons, rrn A and rrn B (Sawada *et al.*, 1981). Only one promoter, which was preceded by a putative Pribnow box and a putative -35 recognition region, was found in the rrn A operon. *M. pneumoniae*, which has a single rRNA operon (Amikam *et al.*, 1984), has been reported to have a single promoter with a putative -10 region, but no promoter-like -35 sequence was found (Hyman *et al.*, 1988). In fact, studies from several research groups shown that many mycoplasma rrn operons were preceded by a consensus Pribnow box and start transcription with a purine as in other bacteria, while the -35 regions bear no resemblance to consensus -35 recognition sequences. Instead, the -35 regions are very A+T rich (Hyman *et al.*, 1988; Rasmussen *et al.*, 1987; Taschke and Herrmann, 1986). The promoter of the single rRNA operon of *M. pneumoniae* functioned *in vitro* with use of *E. coli* RNA polymerase but no transcripts can be detected *in vivo* (Hyman *et al.*, 1988). In the case of the cloned rrnA operon of *M. capricolum*, the promoters were transcribed by *E. coli* RNA polymerase both *in vitro* and *in vivo* (Gafny *et al.*, 1988). This difference may be due to the presence of a -35 region in the promoter of the rrnA operon of *M. capricolum* resembling that of eubacterial promoters (Glaser *et al.*, 1992). It is apparent that the transcription regulatory elements in mycoplasmas are different from those in *E. coli* and other eubacteria. Knudtson and Minion (1992) developed a reporter gene system to examine mycoplasma transcription regulatory components *in vivo*. The test system relied on a translational fusion with trp-lacZYA in which the translational elements were from *E. coli* but promoter function was supplied by either the *M. capricolum* rrnB P2 or the *E. coli* rrnB p1 promoter. They suggested that the A+T rich mycoplasma sequences had pseudopromoter activity in *E. coli* because only 10% of the lacZ fusions active in *E. coli* were also active in *M. pulmonis.*

An unusual structural organization has been found in *M. hyopneumoniae*, which has a single set of rRNA genes. The 16S and 23S rRNA genes of the organism are closely spaced in one operon and the 5S rRNA gene is separated from this operon by at least 4 kbp and may be transcribed separately (Taschke *et al.*, 1986). Transcription of the 16S-23S operon is initiated from either of two tandemly arranged promoters leading to a large precursor RNA consisting of both 16S and 23S rRNA. This primary transcript is first cleaved within stem structures surrounding the two rRNA to yield premature 16S and 23S rRNA. By further processing events the mature 5' and 3' ends are generated. The promoter sequences of this operon differ from those of other eubacterial promoters in lacking the typical -35 region (Taschke and Herrmann, 1986). The putative termination site at the 3' end of the operon is reminiscent of Rho-independent terminators in *E. coli.*

Different organization has also been reported for *Mycoplasma gallisepticum*, in which one locus contains 16S, 23S, and 5S rRNA genes; a second contains 23S and presumably 5S rRNA genes; and a third appears to have only a 16S rRNA gene (Chen and Finch, 1989). Recently, Scamrov and Beabealashvilli found the existence of three regions hybridizing with 16S rRNA and two regions hybridizing with 23S and 5S rRNA in *M. gallisepticum* strain S 6 genome thus forming 4 separate contiguous regions. One set of putative rRNA genes is organized classically in the order 5'-16S-23S-5S-3'. The other two 16S rRNA and the other one 23S-5S rRNA hybridizing regions are separated from each other and from the complete rRNA operon by a distance of more than 6 kbp (Scamrov and Beabealashvilli, 1991).

1.9 Application of DNA restriction and hybridization pattern analysis to the study of genetic relatedness among mycoplasmas and the organization of mycoplasma rRNA genes.

Identity of an organism is inherent in the sequence of the nucleotide bases in its genome, and therefore the proportion of nucleotide base sequences held in common by two organisms indicates the extent of their genetic relatedness. Because direct comparison of base sequences of entire mycoplasmal genomes is still impractical, estimation of the proportion of common base sequences has been made indirectly by a variety of DNA-DNA or DNA-RNA hybridization techniques.

Availability of restriction endonucleases recognizing specific nucleotide sequences in DNA molecules has led to development of another possible approach for assessing genetic relatedness among mollicutes. The cleavage patterns produced by electrophoresis of the digestion products of mycoplasmal DNA resulting from restriction endonuclease treatment provided valuable information on the type and number of specific nucleotide sequences, as well as methylated bases in the mycoplasma chromosome (Razin, 1985) and can be considered genomic fingerprints, known also as restriction fragment length polymorphisms (RFLPs). The use of *Eco*R I cleavage patterns as a aid in mycoplasma taxonomy was first proposed by Bove and associates (Bove and Saillard, 1979, Bove *et al.,* 1982). Since then several research groups have applied the restriction enzyme approach to study the genetic relatedness among mollicutes as well as to detect and to identify mycoplasmas infecting cell cultures (Darai *et al.,* 1981, 1982, 1983; Razin *et al.,* 1983a, b, c). The restriction patterns are particularly effective in determining genetic homogeneity or heterogeneity of strains within established mollicutes species (Razin, 1992; Razin *et al.,* 1983b, c), such as *U. urealyticum* (Razin and Yogev, 1986), *M. hominis* (Christiansen and Andersen, 1988; Yogev *et al.,* 1988a), *A. laidlawii* (Yogev *et al.,* 1988a), *M. capricolum, M. mycoides* (Christiansen and Erno, 1990), *M. gallisepticum, M. synoviae* (Kleven *et al.,* 1988; Yogev *et al.,* 1988b), and *M. pneumoniae* (Yogev *et al.,* 1988a). However, this approach has several major deficiencies. For example, Woese *et al.* (1980) suggested that mycoplasmas are in a state of rapid evolution, brought about by high mutation rates. Because of the high mutation rates, a few base pair differences in restriction enzyme recognition sites might cause the cleavage patterns to change accordingly. The inconsistent presence of extra chromosomal DNA (plasmid, viruses) might also introduce variability in the cleavage patterns (Bove *et al.,* 1982). In addition, although cleavage patterns may be used for determining identity, close similarity, or nonidentity of strains, it is difficult to determine the degree of relatedness on the basis of the patterns, as cleavage data are not readily expressed numerically (Razin *et al.,* 1983b, c). Chan and Ross (1984) reported that better separation of low-molecular-weight DNA fragments was obtained on agarose gels with digestion of the enzymes that recognize G+C rich sequences. However, their approach was limited because it was difficult to separate DNA molecules larger than 20 kbp with conventional electrophoresis. The introduction of the pulsed field gel electrophoresis technique to separate the larger DNA molecules, up to 1000 kbp, provides an useful tool to solve this problem.

One alternative to solve these problems is to compare the hybridization patterns with some well conserved gene probes instead of comparing the cleavage patterns of the whole chromosomal DNA from these organisms. Ribosomal RNA is one of the most conserved classes of macromolecules found in nature (Gutell *et al.,* 1985). The rRNA genes are highly conserved and the best-characterized mycoplasmal genes (Razin, 1985). The fact that the rRNA operons of various mycoplasmas differ in restriction sites within the operon and in their flanking sequences (Amikam *et al.*, 1984; Hyman *et al.*, 1988) leads to hybridization patterns specific to different mycoplasma species, a finding used to identify mycoplasmas in infected cell cultures (Razin *et al.*, 1984a). The hybridization patterns with the rRNA probes might provide some information about the genetic relatedness among mollicutes.

Hybridization using defined rRNA probes to restriction enzyme-digested mycoplasmal genomic DNA has been an useful tool for studying the rRNA genes and has enabled an estimation of rRNA gene copy number and the order of the rRNA genes in mollicutes. As mentioned before, all species of mollicutes tested so far, including plant and insect noncultivable mycoplasma-like organisms (MLOs), carry only one or two sets of rRNA genes in their genomes (Amikam *et al.*, 1982, 1984; Razin, 1985; Razin *et al.*, 1983a, 1984b; Kirkpatrick *et al.,* 1990; Lim and Sears, 1989). Hybridization studies also led to the conclusion that, in mycoplasmas, most rRNA gene sets are linked in the classical order found in other prokaryotes: 5'-16S-23S-5S-3' (Glaser *et al.*, 1984; Gobel *et al.*, 1984; Sawada *et al.*, 1984).

1.10. The model of the secondary structure of the 5S rRNA.

In the last decade, it has become common to sequence nucleic acids (Maxam and Gilbert, 1977; Sanger *et al.*, 1977). Where once it was difficult to sequence a single gene, now portions of genomes containing millions of nucleotides have been sequenced (Turner *et al.*, 1988). The sequence of a genome contains most of the information required to make a functioning organism. The procedure for determining the sequences of RNA and protein molecules that will be made by an organism is relatively straightforward once the genomic sequence is known. How these molecules function, however, depends upon their three-dimensional structure. The first step toward predicting the three-dimensional structure of an RNA is to predict its secondary structure, *i.e.* which nucleotides form base pairs. Several methods are used for predicting secondary structure. One involves phylogenetic comparison, the comparison of sequences with identical function in different organisms (Woese, 1987). This method depends on finding structural features that are conserved during evolution. The second method uses thermodynamics to compare the free energy changes, ΔG, predicted for formation of possible secondary structures (Turner *et al.*, 1988). Computers have been widely used to measure free energy. Experimental data from partial nuclease digestions and proton magnetic resonance spectroscopy provided support for the secondary structure as well (Douthwaite *et al.* 1983; Kan *et al.* 1983).

Many complete sequences are now available for the rRNA from organisms representing a wide phylogenetic spectrum. This data bank has made possible elucidation of the secondary structures of the rRNA by comparative sequence analysis and identification of highly conserved as well as phylogenetically variable regions of these molecules. The secondary structures begin to suggest details of the molecular organization of the rRNA, outlining major structural domains and placing certain constraints on tertiary folding. Clues to the three-dimensional structure of rRNA in the ribosome are begin to emerge and it is now reasonable to expect that about 70% of the helices in an RNA secondary structure will be predicted correctly from a single sequence. However, we are not yet at the stage where a detailed model can be built because of our limited knowledge of the properties of loops (Noller, 1984; Turner *et al.,* 1988).

5S rRNA from *E. coli* and other organisms have been the target of intensive research for more than a decade. The studies performed so far have been concentrated on two aspects: structure and function. A direct involvement of the 5S rRNA in ribosomal function might be anticipated due to the following notions: (1) the presence of 5S rRNA and the direct binding proteins is a prerequisite for *in vitro* reconstruction of functional ribosomes (Dohme and Nierhaus, 1976); (2) an exposed nucleotide sequence of 5S rRNA is strongly shielded from chemical modification when ribosomes contain bound tRNA, demonstrating either a close spatial neighborhood to the tRNA-binding site or a direct participation in the binding process (Goringer *et al.*, 1984); (3) 5S rRNA undergoes a structural alteration when tRNA is bound to the ribosome (Goringer and Wagner, 1988). At this writing, nucleotide sequences for over sixty 5S rRNA have been completed which provided a good base for structural comparison of the elements conserved during evolution as well as the phylogenetic variable elements (Glaser *et al.*, 1992; Hori and Osawa, 1979; Rogers *et al.,* 1985; Taschke *et al.*, 1986).

Determination of secondary structure, even in a relatively small rRNA molecule such as 5S rRNA, can be surprisingly difficult. The most convincing proof for the existence of biologically significant pairing comes from comparative sequence analysis. Briefly, the comparative approach begins with the assumption that the molecules under comparison have essentially the same secondary structure. The primary structures are then aligned, using positions of high sequence homology as a guide. Next, base changes between the compared sequences are noted, and where compensating base changes maintain complementarity between two potential pairing regions, this is taken as evidence for the existence of a true helix at that position (Noller, 1984).

The 5S rRNA species studied so far assumed basically the same secondary structure, in accordance with that proposed previously (Hori and Osawa, 1979; Delihas *et al.* 1984). According to the sizes and the secondary structure, the known 5S rRNA species were suggested to be classified into three types: eukaryotic type (120 bp), prokaryotic 120N type (120 bp, Gram-negative), and prokaryotic 116N type (116 bp, Gram-positive) (Hori and Osawa, 1979). The models of the secondary structures of the types of 5S rRNA are shown in Fig. 4.3 and Fig. 4.4. The data from mycoplasma species indicate that they have distinctly shorter 5S rRNA (Rogers *et al.*, 1985; Taschke *et al.*, 1986).

1.11. The concept of field-inversion gel electrophoresis.

In conventional electrophoresis, using a constant electrical field, DNA molecules behave as "free draining species" (Cantor *et al.* 1988). In free solution all linear DNA molecules have the same mobility, independent of size. In a gel, high resolution size fractionation can be achieved because of the sieving properties of the gel matrix. Polyacrylamide gels are applicable for DNA from a few base pairs to few kilobase pairs in size, while agarose gels work best in the 0.2-20 kbp size range. Here I limit my discussion to agarose gels. Directed by the electrical field, small DNA molecules will move through the gel in a relatively straight path, while large DNA must take a more indirect path to find gel pores large enough to accommodate it . For small DNA molecules, sieving is the major mechanism that provides separation. Thus mobilities are observed to vary logarithmically with DNA size (Fangman, 1978; Cantor and Schimmel, 1980; Cantor *et al.*, 1988; Serwer, 1989).

The sieving properties of a gel become irrelevant once a molecule is longer than the diameter of the largest gel pores. For DNA in agarose, sieving properties are lost when the size exceeds about 20 kbp. Larger DNA molecules exhibit nearly size-independent mobilities, a phenomenon that is thought to be associated with an end-on, reptile-like mode of migration known as "reptation" (Fangman, 1978). The movement of these larger DNA molecules depends strongly on the electrical field strength and the gel concentration because the DNA molecules tend to remain oriented along the direction of the applied electrical fields (Cantor *et al.*, 1988; Hervet and Bean, 1987; Hurley, 1986; Stellwagen, 1985). It is difficult to achieve useful resolution of DNA molecules larger than 50 kbp in conventional electrophoresis. Even in the 10-50 kbp size range, the separation power of ordinary gel electrophoresis is limited. It can be enhanced by operation at very low field strengths and gel concentrations, but these are not very satisfactory experimental conditions because of the long running times required and the mechanical difficulties involved in handling very dilute gels (Fangman, 1978).

Schwartz and Cantor (1984) addressed this problem by employing a complex agarose electrophoresis system which utilized perpendicularly oriented, non-uniform, alternately pulsed electrical fields. The idea behind this method was to force large molecules to turn corners periodically in the gel matrix, thereby introducing a source of size-dependence that is absent during simple migration in one dimension. In typical pulse-field gel electrophoresis experiments, DNA molecules are present in an ordinary agarose gel matrix in conventional low-ionic strength buffers. An electrical field is applied alternately in two directions. To migrate through the matrix, the DNA molecules must change their direction of motion in response to the changing electric field. Separation is achieved because the time required to change direction depends on the size of the DNA. In most cases the apparent reorientation time increases with DNA size; larger molecules reorient more slowly than smaller ones.

Since the original demonstration of the pulse-field gel electrophoresis effect, many different experimental variations on the technique have appeared (Cantor *et al.*, 1988). Although the fundamental physics behind these separations should be the same, the different methods produce somewhat different experimental results. Almost every variation of pulse-field gel electrophoresis has been given a different name. Three basic experimental configurations are in wide use today:

1. Two nominally perpendicular non-homogeneous fields are alternated with equal pulse times (Schwartz and Cantor, 1984). Because of the field non-homogeneity, the angles between the two applied fields vary throughout the gel matrix. Use of this configuration has been called orthogonal field alternating gel electrophoresis.

2. Two nominally homogeneous fields are altered with equal pulse times at a constant angle. Techniques using this configuration have been called voltage clamp (CHEF) (Chu *et al.*, 1986; Vollrath and Davis, 1987), voltage ramp (Cantor *et al.*, 1988), and cross field or swinging gel electrophoresis (Southern *et al.*, 1987), or rotating gel electrophoresis (Serwer, 1987), depending on the method used to alternate the fields.

3. Two nominally homogeneous fields at 180°C are alternated in either duration or strength (Carle *et al.*, 1986). Use of this configuration has been called field inversion gel electrophoresis (FIGE)

All of these different pulse-field gel electrophoresis configurations can be used to separate DNA molecules up to one megabase in size, and some experimental conditions allow the fractionation of molecules that are an order of magnitude larger.

1.12. The application of field-inversion gel electrophoresis in studying the genome of mollicutes.

Since 1984, the field-inversion gel electrophoresis technique has been widely used to study many previously uncharacterized DNA molecules, including the intact (but linearized) chromosomal DNA molecules from many prokaryotes and eukaryotes, *e.g.* physical mapping and size determination of the genomes of *E. coli* K12 (Smith *et al.*, 1987).

Until recently, the construction of genetic maps and the determination of microbial genome sizes were time-consuming procedures. Genetic linkage maps, like the one constructed for *E. coli* (Bachmann, 1990), were assembled from analysis of conjugational recombination, complementation, generalized transduction data, and mapping of transposon insertions. DNA size measurements were performed by renaturation kinetics or electron microscopy techniques (Bak *et al.*, 1969; Bode and Morowitz, 1967; Chow, 1977; Gillis *et al.*, 1970).

Developments in the preparation and separation of large DNA fragments have made it possible to devise relatively simple schemes to map the DNA structure of the entire genome (Schwartz and Cantor, 1984). Pulsed-field gel electrophoresis (PFGE, Cantor *et al.*, 1988; Schwartz and Cantor, 1984, Smith and Cantor, 1986) enabled high resolution size separation of the large DNA fragments resulting from *in situ* digestion of unbroken DNA with "rare-cutting" restriction enzymes, i.e. enzymes recognizing a restriction site expected to occur only rarely (10 to 50 times) in the genome under investigation. The resulting individual restriction fragments could then be linked by standard techniques, such as multiple digestions with different enzymes, cloning of linking DNA fragments (Kohara *et al.*, 1987; Wenzel *et al.*, 1992), or various two-dimensional agarose gel systems (Bautsch, 1988; Romling *et al.*, 1989; Yee and Inouye, 1982).

The only precondition for DNA size analysis by pulse-field gel electrophoresis is that the bacterial DNA must be linear in order to be separated according to size. Circular genomes can be linearized by partial digestion with infrequently cutting restriction enzymes or by controlled gamma irradiation (Ostashevsky and Lange, 1987). Alternatively, genomes are digested to completion with a suitable restriction enzyme, and the sizes of the individual fragments are determined by PFGE and added up to obtain the total genome size.

The mollicutes, possessing the smallest bacterial genomes known, are particularly suited for the analysis by PFGE. Numerous new research activities in the area have led to the size determination of many mycoplasma genomes (Neimark and Lange, 1990; Robertson *et al.*, 1990) and to the construction of an increasing number of physical and genetic maps (Bautsch, 1988; Cocks *et al.*, 1989; Colman *et al.*, 1990; Krause and Mawn, 1990; Pyle and Finch, 1988; Pyle *et al.*, 1990; Whitley *et al.*, 1991).

1.13. Research objectives.

One objective of this study was to compare the rRNA gene organization of two porcine mycoplasma species, *M. hyopneumoniae* and *M. flocculare*. The study has then been extended to another porcine mycoplasma species, *Mycoplasma hyorhinis* and two strains of a human mycoplasma species, *Mycoplasma fermentans*, which has been found in some AIDS patients. We believe that broader scale study of the organization of the rRNA genes of the mollicutes and other eubacteria may help to understand the evolutionary relationship between the mollicutes and eubacteria as well as among mollicutes themselves.

As mentioned, the genome of most mollicutes carries only one or two copies of rRNA genes (Amikam *et al.*, 1982, 1984) which may facilitate studies on the control mechanisms of rRNA synthesis. Taschke *et al.* (1986) have reported an unusual structural organization of rRNA genes in *M. hyopneumoniae*. Since *M. hyopneumoniae*, *M. flocculare*, and *M. hyorhinis* are three porcine mycoplasma species isolated from the respiratory tract and previous studies have shown that *M. hyopneumoniae* and *M. flocculare* are more closely related to one another than other mycoplasma species, comparing the rRNA gene organization among these organisms can provide useful information about their phylogenetic relationship.

Another objective of this study was to compare the physical maps of the *M. flocculare* and *M. hyopneumoniae* genomes. Constructing the physical map is the first step towards constructing the genetic map to study the genetic structure of the mycoplasma genomes. It will be interesting to compare the physical maps as well as the genetic maps of these two closely related mycoplasma species. Any differences in their genomic structure might be useful for studying their genetic relatedness. This study was then extended to *M. hyorhinis* and two "*M. fermentans* strains".

The third objective was to investigate the transcription regulation elements of the *M. flocculare* 5S rRNA gene. I have demonstrated that *M. flocculare*, like *M. hyopneumoniae*, has a single set of each rRNA gene (16S, 23S, and 5S) and that its 5S rRNA gene is separated from the 5'-16S-23S-3' rRNA gene cluster by a distance of more than 150 kbp. It is possible that the transcription of these separated rRNA loci is regulated by their own regulatory elements or that they could be cotranscribed with other genes into long precursors and then processed to mature rRNA species. I decided to clone and sequence the *M. flocculare* 5S rRNA gene and its flanking regions to look for transcription regulatory elements. This will allow further study of the transcription regulation mechanism.

CHAPTER II. MATERIALS AND METHODS

2.1 Chemicals and enzymes.

Chemicals were purchased from Sigma Chemical Co. (ST. Louis, Mo., U. S. A.), BDH Inc. (Poole, England), Fisher Scientific Company (Fair Lawn, N. J., U. S. A.), Eastman Kodak Co. (Rochester, N. Y., U. S. A.), Aldrich Chemical Co. (Milwaukee, Wis. U. S. A.), J. T. Baker Chemical Co. (Philipsburg, N. J., U. S. A.), and Boehringer Mannheim Canada (Dorval, Que., Canada). Nitrocellulose filters were from Amersham (Burkinghamshire, England) and Bio-Rad Laboratories (Richmond, Calif. U. S. A.). Kodak XAR5 X-ray film was obtained from Eastman Kodak Co., through Innomed Imaging. Most media were from DIFCO Laboratories (Detroit, Mich., U. S. A.) except mycoplasma Friis medium which was prepared by myself.

Nucleotides were purchased from Pharmacia (Uppsala, Sweden) and Boehringer Mannheim Canada. Radioisotopically labeled compounds, including ^{35}S-dATP, α-^{32}P-dATP, and γ-^{32}P-ATP, were obtained from ICN Biochemicals Inc. (Cleveland, Ohio, U. S. A.).

The restriction endonucleases were obtained from Bethesda Research Laboratory Inc. (Gaitherburg, Md., U. S. A.), Boehringer Mannheim Canada, Pharmacia, and New England Biolabs Inc. (Mississiauga, Ontario, Canada) (Table 2.1). T4 DNA ligase and the Klenow fragment of *E. coli* DNA polymerase I were from Boehringer Mannheim Canada; avian myeloblastosis virus (AMV) reverse transcriptase and T4 polynucleotide Kinase were from Pharmacia. T7 DNA polymerase was from Pharmacia. All enzymes were used as recommended by the manufacturers.

Table 2.1 Restriction enzymes used in this study.

Enzymes	**Recognition sequences**	**Suppliers (Cat. No.)**
Alu I	AG/CT	Bethesda Research Laboratories (5200SA)
Apa I	GGGCC/C	Boehringer Mannheim Canada (899208) Bethesda Research Laboratories (5440SA)
Apa L I	G/TGCAC	New England Biolabs (#507S)
*Asp*718 I	G/GTACC	Boehringer Mannheim Canada (814245)
Bam H I	G/GATCC	Boehringer Mannheim Canada (220612) Bethesda Research Laboratories (5201SA) New England Biolabs (#136S) Pharmacia (27-0868-03)
Bgl I	GCCNNNN/NGGC	Boehringer Mannheim Canada (404101) Pharmacia (27-0944-01)
Bgl II	A/GATCT	Bethesda Research Laboratories (5219SA)
Bss H II	G/CGCGC	Boehringer Mannheim Canada (1168851) New England Biolabs (#199L)
Bst E II	G/GTNACC	Bethesda Research Laboratories (5225SA) Boehringer Mannheim Canada (404233)
Cla I	AT/CGAT	New England Biolabs (#197S)
Dra I	TTT/AAA	Bethesda Research Laboratories (5430SA)
Eco R I	G/AATTC	Bethesda Research Laboratories (5202SA)
Eco R V	GAT/ATC	Bethesda Research Laboratories (5425SA)
Hin d III	A/AGCTT	Bethesda Research Laboratories (5207SA)
Hpa I	GTT/AAC	Bethesda Research Laboratories (5208SA)
Kpn I	GGTAC/C	Boehringer Mannheim Canada (899186)
Ksp I	CCGC/GG	Boehringer Mannheim Canada (1117807)
Mbo I	/GATC	Bethesda Research Laboratories (5248SA)
Mlu I	A/CGCGT	Boehringer Mannheim Canada (909700)
Nae I	GCC/GGC	Boehringer Mannheim Canada (786314)
Nar I	GG/CGCC	Boehringer Mannheim Canada (1103016)
Ngo M I	G/CCGGC	New England Biolabs (#564S)
Not I	GC/GGCCGC	Boehringer Mannheim Canada (1014706) Pharmacia (27-0976-01)
Pst I	CTGCA/G	Boehringer Mannheim Canada (621625)
Pvu I	CGAT/CG	Boehringer Mannheim Canada (650137)
Pvu II	CAG/CTG	Boehringer Mannheim Canada (642690)
Sac I	GAGCT/C	Pharmacia (27-0970-01)
Sac II	CCGC/GG	Pharmacia (27-0910-01)
Sal I	G/TCGAC	Boehringer Mannheim Canada (348783)

Enzymes	**Recognition sequences**	**Suppliers (Cat. No.)**
Sau 3A	/GATC	Bethesda Research Laboratories (5229SA) Boehringer Mannheim Canada (709473)
Sca I	AGT/ACT	Bethesda Research Laboratories (5436SA)
Sno I	G/TGCAC	Boehringer Mannheim Canada (1088548)
Stu I	AGG/CCT	New England Biolabs (#132S)
Sty I	C/CAAGG or C/CTTGG	Boehringer Mannheim Canada (1047744)
Xho I	C/TCGAG	Boehringer Mannheim Canada (899194)
Xba I	T/CTAGA	Bethesda Research Laboratories (5226SA)

Table 2.2 Primers used in this study.

Oligonucleotides	Sequences
Mhp5S17mer (1)	5'-TGAATCCATTCCGAACT-3'
Mhp16S19mer (1)	5'-GATCCTGGCTCAGGATAAA-3'
YH3 (1)	5'-AGCCAATTTTTGGGACA-3'
YH4 (1)	5'-TTTTTTTACATTTTCTAT-3'
YH6 (1)	5'-ACTATGGTAAGGCTTGAG-3'
GWS 7 (Mf5S) (1)	5'-CTTTTCCGGCGTTTTGT-3'
GWS 51 (Mf 23S) (1)	5'-CCTGGAGCTGAAGTC-3'
GWS 60 (Mf 23S) (1)	5'-GCGACTCGAGAAAGTCGATGAAGGACGT-3'
Universal primer (2)	5'-GTAAAACGACGGCCAGT-3'
Reverse sequencing primer(2)	5'-CAGGAAACAGCTATGAC-3'
Hybridization primer (2)	5'-CACAATTCCACAAAC-3'

(1) Prepared by Dr. K. L. Roy of Department of Microbiology, University of Alberta.

(2) From Boehringer Mannheim Canada.

M13 universal sequencing primer, hybridization primer, and reverse primer were purchased from Boehringer Mannheim Canada. Random primer and all other oligonucleotides used in this study were made by Dr. K. L. Roy of Department of Microbiology, University of Alberta, using a model 381A DNA synthesizer from Applied Biosystems Canada Inc. (Mississiauga, Ontario, Canada).

2.2. Bacterial Strains, plasmids and bacteriophages.

M. hyopneumoniae ATCC 25095, *M. flocculare* ATCC 27716, *M. hyorhinis* ATCC 17891, and *M. fermentans* ATCC 19989 were obtained from the American Type Culture Collection (Rockville, MD, U.S.A). Two individual batches of *M. fermentans* ATCC 19989 cells, Mfer-A (culture of Oct. 5, 1985) and Mfer-B (culture of Apr. 4, 1992), were gifts from Dr. J. A. Robertson (Department of Medical Microbiology and Infectious Diseases of University of Alberta). *M. fermentans* (*incognitus* strain) was a gift from Dr. S. -C. Lo (Armed Forces Institute of Pathology, Washington, D. C.) and cultivated by Dr. J. A. Robertson.

E. coli DH5αF' strain was purchased from Bethesda Research Laboratory; *E. coli* LE 392 and JM101 strains were from Stratagene (La Jolla, California, U. S. A.).

M13mp19 was obtained from Boehringer Mannheim Canada; pBluescript I was from Stratagene; λ cI 857 Sam7 was from Boehringer Mannheim Canada.

2.3. Growth of mycoplasmas.

M. hyopneumoniae ATCC 25095, *M. flocculare* ATCC 27716, and *M. hyorhinis* ATCC 17891 were grown in a non-selective broth medium based on Friis' formulation (Friis, 1975). A volume (2 ml) of Friis' medium was inoculated with 10 μl of mycoplasma culture and incubated for 24-48 hours at 37°C to mid-log phase (2-5x10^7

cells/ ml), indicated by a slight orange-yellow color change of the medium or by measuring changes in the hydrogen ion concentrations with a pH meter. The cultures were then used as inoculum to inoculate 100 ml of Friis' medium and incubated under the same condition as described above. The cells were harvested by centrifugation at 33,000 g for 25 min. in the cold, washed and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA)(Stemke *et al.,* 1985).

2.4. Growth of *E. coli*:

E. coli was routinely grown in 2 x YT broth (1.4% tryptone, 1% yeast extract, 0.3% NaCl) (Sambrook *et al.* 1989) or 2 x YT agar (1.4% tryptone, 1% yeast extract, 0.3% NaCl, 1.5% agar). LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) was also used. When selection was required, ampicillin was added to the medium (Ayerst Laboratories, Montreal, Canada).

2.5 Genomic DNA isolation.

A 100 ml culture of the mycoplasma (equivalent to 10^{10} cells) was centrifuged, the cells were washed, and resuspended in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.2% SDS, 20 µg/ml Proteinase K. The solution was incubated at 50°C for 2 hours, extracted twice with 1:1 chloroform/ phenol and once with chloroform. The DNA samples were precipitated by adding sodium acetate to a concentration of 0.3 M and 2 volumes of 95% ethanol were added to the aqueous phase. The solution was kept in a -20°C freezer overnight. After centrifugation, the moist pellet was resuspended in 100 µl TE buffer and stored in the -20°C freezer. Genomic DNA samples from *E. coli* were purified by the method of Sambrook *et al.* (1989).

2.6. Total RNA purification.

Total RNA from *E. coli* was purified according to the procedure described by Gafny *et al.* (1988). Cells were grown at 37°C in 125 ml of LB medium (Sambrook *et al.,* 1989) to an OD_{600}=0.7, poured onto ice, collected by centrifugation and resuspended in 10 ml of 10 mM Tris-HCl, pH 7.3, containing 10 mM KCl, 5 mM $MgCl_2$, and 3 mg lysozyme. The cells were then lysed by freezing (-70°C) and thawing at 64°C. Sodium acetate (pH 5.2) was added to a final concentration of 0.1 M, followed by two extraction with TE-saturated phenol at room temperature. The RNA in the aqueous phase was precipitated with two volumes of 95% ethanol at room temperature. The pellet was resuspended in 100 μl of TE buffer and stored at -20°C.

To isolate *M. flocculare* total RNA, the pellet from a one litre culture was resuspended in 5 ml of 10 mM Tris-HCl, pH 7.3, containing 10 mM KCl, 5 mM MgCl, and 1% SDS. The cells were then lysed by freezing at -70°C and thawing at 64°C. After two extraction with TE-saturated phenol and one extraction with chloroform, the RNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol. The pellet was dissolved in 100 μl of TE buffer and stored at -20°C.

2.7. Primer extension analysis.

An *E. coli* or *M. flocculare* total RNA sample (3 μg) was mixed with 20 ng of 5'-end-labeled oligonucleotide primer, YH 6 (see section 2.21), synthesized according to the phosphoramidite method (Matteucci, 1981) by Dr. K. L. Roy from Dept. of Microbiology, Univ. of Alberta. Sodium acetate (pH 5.5) was added to a concentration of 0.3 M and then 2 volumes 95% ethanol were added to the solution and left in the -20°C freezer overnight. After centrifugation at 4°C, the pellet was resuspended in 20 μl of 0.1 M Tris-HCl, pH 8.3, with 0.14 M KCl and 0.01 M $MgCl_2$. The resulting solution was incubated at 65°C for 10 min. and cooled to room temperature for 5 min. Dithiothreitol was added to a final concentration of 0.01 M and the four deoxynucleotide triphosphates were added to a final concentration of 1 mM. Reverse transcriptase (18 units) of AMV (Pharmacia) was added and the mixture was incubated at 37-45°C for one hour. The reaction was stopped by heat inactivation at 75°C for 10 min. DNase-free RNase (1 μg) was added and incubation continued for 30 min. at 37°C. After one extraction with an equal volume of TE-saturated phenol, ammonium acetate was added to a final concentration of 0.4 M followed by ethanol precipitation overnight at -20°C. The precipitate was resuspended in 2 μl of 98% formamide, 0.1% bromophenol blue, 0.1% xylan cyanol, 2 mM EDTA and run on a sequencing gel.

2.8. Cloning of the *M. flocculare* 5S rRNA gene.

M. flocculare DNA samples were digested by restriction enzyme *Xba* I (BRL, Gaithersburg, Maryland, U.S.A.) under the conditions recommended by the manufacture and the *Xba* I fragments were ligated into the M13 mp19 vectors (Boerhinger Mannheim). The recombinant DNA samples were then transformed into *E. coli* JM 101 by the method of Morrison (1979). For screening the recombinant libraries, the plaques were transferred to nitrocellulose filters, denatured in 1.5 M NaCl, 0.5 M NaOH and neutralized in 20x SSPE, 0.5M Tris-HCl pH 5.5. After baking at 70°C under vacuum for 2 hours, the filters were prehybridized in prehybridization solution (6x SSPE, 5x Denhardt's, 0.5% SDS), and 0.03 mg/ml denatured salmon sperm DNA for 3 hours at 60°C and hybridized in prehybridization solution with 20% formamide for 24 hours with γ-^{32}P-ATP labeled 5S rRNA specific oligonucleotide probe, Mhp5S17mer, based upon the *M. hyopneumoniae* 5S rRNA sequence. Several possible positive M13 recombinant clones were chosen for further analysis. After amplifying and replanting of those recombinant clones, only one clone was confirmed to be positive. This positive M13 recombinant clone was repurified three times and stored at 4°C.

2.9. Isolation of M13 recombinant single stranded and double stranded Rf DNA.

The colonies of an *E. coli* strain carrying an F' episome (JM 101) were picked from a minimal (M9) agar plate (Sambrook *et al.*, 1989) or colonies of *E. coli* strain dH5 αF' were picked from a 2YT plate (Sambrook *et al.*, 1989). These colonies were then inoculated in 2YT broth, and incubated overnight at 37°C.

Mini-preparations of single stranded DNA were carried out as following. A volume (2 ml) of the diluted overnight culture (1:50 in 2YT) was inoculated with M13 recombinant and incubated at 37°C with very vigorous shaking for 4.5 hours. After centrifugation, M13 bacteriophage was precipitated by adding 250 µl of 20% PEG/ 3.5 M ammonium acetate solution to 1 ml of supernant and incubating on ice for 30 min. The pellet was dissolved in 100 µl of TE buffer, followed by two extractions with TE-saturated phenol and one extraction with chloroform at room temperature. The single stranded DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol at -20°C overnight. The DNA samples were then stored in 50 ml of TE buffer at -20°C.

Preparation of the M13 bacteriophage Rf DNA was performed according to Sambrook *et al.* (1989) with some modifications. In brief, a volume (50 ml) of the diluted overnight culture (1:50 in 2YT broth) was inoculated with 100 µl of M13 bacteriophage solution (10^{11} phage particles/ml) and incubated at 37°C with very vigorous shaking for 4 hours. The infected culture was then used to inoculate 2 L of the diluted overnight culture and incubated under the same condition for 4 hours. The cells, collected by centrifugation, were resuspended in 40 ml of 25 mM Tris-HCl, pH 8.0, containing 50 mM glucose, 10 mM EDTA, and 40 mg of lysozyme. Following addition of 80 ml of 0.2 N NaOH containing

10% SDS, the suspension was incubated at room temperature for 10 min. Potassium acetate and glacial acetic acid were then added to a final concentration of 3 M and 5 M, respectively, and incubated on ice for 10 min. The M13 DNA molecules in the aqueous phase were precipitated with 0.6 volume of isopropanol at room temperature. After centrifugation, the pellet was dissolved in TE buffer and stored at -20°C. Two methods were then used to further purify the M13 Rf DNA samples.

Small scale M13 Rf DNA preparation was carried out as following: 0.6 ml of M13 DNA, which was prepared as described above, was mixed with an equal volume of 13% PEG containing 1.6 M NaCl (Lis, 1980). After incubating at 4°C overnight, the pellet was dissolved in 250 µl of TE buffer and extracted twice with an equal volume of phenol/chloroform (1:1). The M13 Rf DNA was precipitated with 0.1 volume of sodium acetate and 2 volumes of 95% ethanol. The pellet was then resuspended in TE buffer and stored at 4°C.

Large scale M13 Rf DNA preparation was performed according to Sambrook *et al.* (1989). Briefly, M13 DNA, which was prepared from 2 L of culture as described above, was dissolved in 10 ml TE buffer. A volume (0.8 ml) of ethidium bromide (0.8 mg/ml) and then 10 g of solid CsCl were added to a final density of 1.55 g/ml for the solution. After centrifugation at 30000 x g for 48 hours at 20°C, the band containing M13 Rf DNA was collected and precipitated with 0.6 volume of isopropanol at room temperature. The DNA pellet was dissolved in TE buffer and stored at -20°C.

2.10. DNA sequence analysis.

Single strand DNA sequence analysis was performed according to Sanger's chain termination method (1977) by using the dideoxy sequencing kit (Boerhinger Mannheim). In brief, the single stranded DNA isolated from the recombinant M13 clone was used as template and three primers (the commercial universal primer and the synthesized primers, Mhp5S17mer and YH4) were used for the negative strand DNA sequencing. Another commercial dideoxy sequencing kit (Pharmacia) was employed for double strand sequencing of M13 Rf DNA with the protocol from the supplier. Double stranded DNA prepared from the recombinant M13 clone was also used as template and several oligonucleotide primers, including the commercial reverse sequencing primer and the synthesized Mf5Sdd, were used for the positive strand DNA sequencing.

2.11. Methodology of the computer analysis.

Sequencing files were analyzed using the NIH/DCRT analysis system of M. Kanehisa (IBM PC version). *M. hyopneumoniae* and *M. flocculare* 5S rRNA sequence comparisons and RNA folding were also done using PCGENE (Intellgenetics, Inc., Mountain View, CA, USA).

2.12. Preparation of intact mycoplasma chromosomal DNA and restriction endonuclease digestion.

Mycoplasma cells were grown and harvested as described in this chapter. The cells were incorporated into blocks of low melting agarose (Sigma, St. Louis, MD, U.S.A.) as described by Robertson *et al.* (1990). The blocks were treated with 2 mg/ml protease K in lysis buffer containing 1% Sarkosyl, 0.5M EDTA, and 10 mM Tris/HCl pH 9.5, for 12 hours at 50°C. The blocks were stored in the lysis buffer at 4°C. Before use, the blocks were cut into slices (1x1x10 mm) and rinsed several times in large volumes of TE buffer at 4°C overnight. The slices were then suspended in 0.1 ml of restriction buffer, 10-30 units of restriction enzyme were added and the mixtures were incubated for 12 hours under the conditions recommended by the suppliers.

2.13. Preparation of the size markers.

Lambda ladders were used as size markers. 2.5 μg of lambda DNA (Boerhinger Mannheim) was mixed with 16 μl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and heated to 70°C for 10 min. and then suspended in 50 μl of 2% low melting agarose in TE buffer to form agarose blocks. The blocks were kept in 0.5 M EDTA for 2 hours and then stored in 50 mM EDTA at 4°C until used. A commercial yeast chromosome size marker (Boerhinger Mannheim) was also used.

2.14 Field-inversion gel electrophoresis:

Field-inversion gel electrophoresis (FIGE) was carried out as follows: intact mycoplasma DNA molecules in low-melting agarose blocks were electrophoresed in 1% agarose gels in 0.5 x TBE (0.4 M Tris-HCl, pH 8.2, 0.4 M boric acid, and 10 mM EDTA)(Pyle and Finch 1988) for 14-18 hours at 12-15°C with a pulse ratio of 2:1 and an electric field strength of 12.5 V/cm, using a Pulsewave 760 Switcher (Bio-Rad) and a home-built electrophoresis apparatus. An exponentially increasing forward switch times from 1 to 10 sec. were used. After the electrophoresis, the gels were stained with ethidium bromide and photographed under ultra-violet light. The DNA fragments in the gels were then transferred to nitrocellulose membranes by the Southern method (Southern, 1975). The membranes were stored at 4°C until use. Some of the DNA fragments were excised for double digestion or preparation of large DNA fragment probes.

2.15 Contour-clamped homogeneous electric fields electrophoresis:

A CHEF-DR II system (Bio-Rad) was used for the clamped homogeneous electric fields gel electrophoresis (CHEF). Intact mycoplasma DNA molecules were incorporated into low-melting agarose blocks, digested with the restriction enzymes and electrophoresed in 0.5 x TBE buffer at 14°C for 20-24 hours with a pulse ratio of 1:1 and an electric field strength of 16 V/cm. Exponentially increasing forward switch times from 5 to 30 sec. were used to separate DNA fragments up to 250 kbp, while the switch times from 30 to 100 sec. were used when the DNA fragments were larger than 250 kbp. After the electrophoresis, the gels were treated the same way as described above.

2.16. Conventional electrophoresis.

Conventional agarose gel electrophoresis was used to separate DNA restriction fragments smaller than 10 kbp in size. After restriction enzyme digestion, DNA samples were electrophoresed in 0.7-1.0 % agarose gels in 0.5 x TBE (0.4 M Tris-HCl, pH 8.2, 0.4 M boric acid, and 10 mM EDTA) or in 1 x TEA (40 mM Tris.acetate, 1 mM EDTA, pH 8.0) for 60-90 min. at room temperature with an electric field strength of 10 V/cm. After the electrophoresis, the gels were treated as described above except that no DNA fragments were excised for double digestion.

2.17. Double restriction endonuclease digestion.

After the first restriction endonuclease digestion and electrophoresis (FIGE or CHEF), the gels were stained with ethidium bromide. Selected portions of the gels (5x2x3 mm) containing DNA restriction fragments were excised under ultraviolet light and washed with large volumes of TE buffer. After the agarose slices were placed in 0.15 ml of 1x restriction buffer, 10-30 units of the proper restriction enzymes were added. The mixtures were incubated for 12 hours under the recommended conditions. The DNA samples were electrophoresed under the same conditions as the single restriction digestion except that 1.2% agarose was employed.

Some of the double restriction digestions were carried out as following: the agarose slices were placed in 0.15 ml of 1x restriction buffer, 10-30 units of restriction enzymes for the first digestion were added and the mixtures were incubated. The slices were then moved to new tubes with proper 1x restriction buffer for the second digestion. The second restriction enzymes were then added and the tubes were incubated under the conditions recommended for the secondary digestion. The electrophoresis was carried out under the same conditions as the single digestion.

2.18. Southern analysis.

Southern transfers (Southern, 1975) were carried out as follows. After electrophoresis (ordinary electrophoresis, FIGE, CHEF), the gels were stained with ethidium bromide for photographing. After photographing, the gels were treated for 1 hour with 1.5M NaCl, 0.5 M NaOH and for 1.5-2.0 hours with 20x SSPE (Sambrook *et al.*, 1989), 0.5M Tris-HCl pH 5.5. DNA molecules was transferred to nitrocellulose membrane (Trans-Blot, Bio-Rad. Richmond, Calif., U.S.A.), prehybridized for 2-4 hours at 60°C and hybridized as described by Sambrook *et al.* (1989). The stringency of the hybridization conditions and the washing conditions after hybridization were chosen conformity to the probes that had been used and the results of the hybridization. For example, the hybridization temperature was 28°C for oligonucleotide probes and 60°C for large genomic DNA fragment probes which had been labeled by random labeling method. The membranes were washed with 2x SSPE, 0.1% SDS at room temperature for oligonucleotide probes and washed with 1x SSPE, 0.1% SDS at 50-60°C for the large restriction fragment probes . The membranes were then exposed to Kodak XAR-5 film at -70°C for up to seven days depending upon the strength of the signals. To strip the probe from the blots, the membranes were washed for 30 s with 1.5 M NaCl, 0.5 M NaOH, and for 1 hour with 20x SSPE, 0.5 M Tris-HCl, pH 5.5 at room temperature. The membranes could be re-used 4-6 times.

2.19. Preparation of large DNA fragments probes.

Intact genomic DNA molecules from *M. hyopneumoniae*, *M. flocculare*, and *M. fermentans* (*incognitus* strain) were digested with different restriction enzymes under the conditions recommended by the suppliers. After electrophoresis (FIGE or CHEF), the portions of the gels with large DNA restriction fragments were excised under ultraviolet light illumination. The DNA samples were then isolated from agarose gels using a commercial Geneclean Kit (Bio 101 Inc., La Jolla , CA, U.S.A.) following the instruction from the supplier. Briefly, the excised DNA fragments were dissolved in "Na I solution" (1 g agarose/1 ml Na I) at 50 °C for 5 min. The solution was mixed with 15 µl of "Glassmilk" and incubated on ice for 10 min. After centrifuging at 21000 x g for 5 seconds, the pellet was washed with "New Wash solution" three times, resuspended in 100 µl of TE buffer (10 mM Tris.HCl, 1 mM EDTA, pH 8.0), and incubated at 50 °C for 10 min. The mixture was again centrifuged under the same conditions and the DNA fragments in the solution were stored at - 20 °C. The DNA fragments were labeled with α-^{32}P-dATP using the random priming method (Feinberg and Vogelstein, 1983, 1984; Sambrook *et al.*, 1989).

2.20. Random primer DNA labeling.

The modified Feinberg and Vogelstein method (1983, 1984) was used to label the large genomic DNA fragments isolated from FIGE or CHEF gels. In short, 16 µl of DNA samples (20-25 µg/ml) were mixed with 1 µl of oligodeoxyribonucleotide primers (hexamers, 50 A_{260}/ml), 6 µl of 4 x NT buffer (200 mM Tris.HCl, pH 7.8, 20 mM $MgCl_2$, 10 mM dithiothreitol, and 200 µg/ml bovine serum albumin, 1 µl of each 1 mM dNTP (dGTP, dTTP, and dCTP), and 10-20 µCi of α- ^{32}P- dATP. The mixture was heated at 90 °C for 2 min. and incubated at 37°C for 15 min. The large fragment of DNA polymerase I (1 or 2 units) was then added and the components were incubated at 37 °C for 2 hours.

2.21. Kinase 5' end labeling.

Kinase 5' end labeling assay was performed according to Donis-Keller *et al.* (1977). Oligonucleotides (1-10 p mol) or hydrolyzed RNA (0.2-0.25 μg/μl) were mixed with 1 μl 10 x kinase buffer (0.5 M Tris.HCl, pH 7.6, 0.1 M $MgCl_2$, and 1 mM EDTA), 10-20 μCi of γ- ^{32}P- ATP, and 5 units of T4 polynucleotide kinase in a total volume of 10 μl. The solution was incubated at 37 °C for 30 min. The probes were directly used directly for the hybridization.

2.22. Preparation of cloned *M. hyopneumoniae* genomic DNA probes.

Two M13 mp19 clones, 37N and 50N, contain inserts of different *Eco* RI fragments from *M. hyopneumoniae* genomic DNA samples (Stemke, 1989). The single stranded M13 DNA from these two clones were labeled with α- ^{32}P- dATP using the universal hybridization priming method (Sambrook *et al.*, 1989) and used as *M. hyopneumoniae* specific probes to detect the corresponding DNA fragments.

2.23. Preparation of the rRNA probes.

Several different rRNA probes were used to determine the location of the rRNA genes on genomic maps. The 16S and 23S rRNA samples were obtained from *Escherichia coli* K12-712 and purified by sucrose gradient density by the method of McConkey (1967). In brief, after preparing total RNA from *E. coli* as described early in this chapter, the RNA samples were loaded onto a 5-40 % sucrose gradient in TCM buffer (10 mM Tris-HCl, pH

7.4, containing 10mM $MgCl_2$) and centrifuged at 60 000 x g for 17-25 hours at 4°C. Different fractions containing the 16S or 23S rRNA molecules were collected. The 16S or 23S rRNA samples were precipitated by adding 0.1 volume of 12 M LiCl and 2 volume of 95% ethanol and storing at -20°C overnight. When used as a probe, the RNA samples were partially hydrolyzed by exposure to 50 mM carbonate/ bicarbonate, pH 9.0, 1 mM EDTA at 90°C for 6-9 min. (Donis-Keller *et al.*, 1977). The fragments were then precipitated by ethanol and labeled using T4 kinase with γ-^{32}P-ATP (Donis-Keller *et al.*, 1977).

pF 1-6, which is a pBluescript plasmid (Short *et al.*, 1988) clone carrying an *Eco*R I fragment about 12 kbp in size from *M. flocculare* genomic DNA, contained the complete *M. flocculare* 16S rRNA gene and 23S rRNA genes (Stemke *et al.*, 1992). Plasmid DNA samples were labeled with α- ^{32}P- dATP using the random priming method (Feinberg and Vogelstein, 1983, 1984; Sambrook *et al.*, 1989).

pR133, which contained a copy of the *Spiroplasma citri* 16S rRNA gene, was kindly provided by Dr. O. Grau (Laigret *et al.*, 1990). Plasmid DNA samples were digested with *Dra* I to release the 16S rRNA gene fragments and then run on an agarose gel. DNA fragments were isolated from the gel using a commercial Geneclean Kit (Bio 101 Inc.) following the instruction from the supplier and labeled with α-^{32}P- dATP using random priming.

M13 Mf 5S, which is a M13 mp19 clone inserted with a 700 bp *Xba* 1 fragment from *M. flocculare* genomic DNA, contained a complete 5S rRNA gene. M13 Mf 5S DNA samples were labeled with α- ^{32}P- dATP using the universal hybridization priming method (Sambrook *et al.*, 1989).

The oligonucleotide, Mhp5S17mer, 5'-TGAATCCATTCCGAACT-3', based upon the *M. hyopneumoniae* 5S rRNA sequence reported by Taschke *et al* (Taschke *et al.*, 1989), was prepared using the phosphoramidite method (Matteucci and Caruthers, 1981). It was labeled with γ- ^{32}P- ATP by the T4 Kinase method (Donis-Keller *et al.*, 1977).

The oligonucleotides GWS 51, 5'-CCTGGAGCTGAAGTC-3', and GWS 60, 5'-GCGACTCGAGAAAGTCGATGAAGGACGT-3', based upon the *M. flocculare* 23S rRNA sequence (Genbank accession #L22210), were obtained as above. These were labeled as described above and used as probes to detect the 23S rRNA genes.

CHAPTER III. RESULTS

3.1. Comparison of the DNA restriction endonuclease cleavage patterns of *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare.*

Digestion of chromosomal DNA preparations from *M. hyopneumoniae* and *M. flocculare* with these restriction enzymes with recognition sites rich in A+T residues could cleave the mycoplasma genomes into large numbers of small DNA fragments. In this study the following enzymes were tested: *Alu* I, *Bgl* II, *Cla* I, *Dra* I, *Eco*R I, *Eco*R V , *Hin*dIII, *Hpa* I, *Mbo* I, *Sau*3A I , *Sca* I, and *Xba* I. After digestion, the DNA samples were size fractionated on 0.8 % agarose gels by conventional electrophoresis. The results obtained after cleavage of *M. hyopneumoniae* and *M. flocculare* DNA preparations with *Hpa* I, *Eco*R V, and *Sca* I are shown in Fig. 3.1. The DNA from these two organisms were sensitive to all the enzymes mentioned above. The reproducibility of the cleavage patterns was demonstrated by the identity of patterns obtained upon testing different DNA preparations from the same mycoplasma strain and by changing the batch of the specific restriction enzyme employed. The high reproducibility of the results can also be taken to indicate that, under the experimental conditions used, the digestion of the mycoplasmal DNA reached completion. Most of the restriction enzymes generated large numbers of restriction fragments and many of the fragments had overlapping sizes. It was difficult to obtain better separation of the restriction fragments because of the large number and small size of the restriction fragments. For the twelve enzymes tested, the distribution patterns for the two species are different with some distinguishing bands, *i.e.* Fig. 3.1, line 2: a band with size about 3750 bp. It was difficult to compare the cleavage patterns of these chromosomal DNA quantitatively to decide the genetic relatedness between the two species.

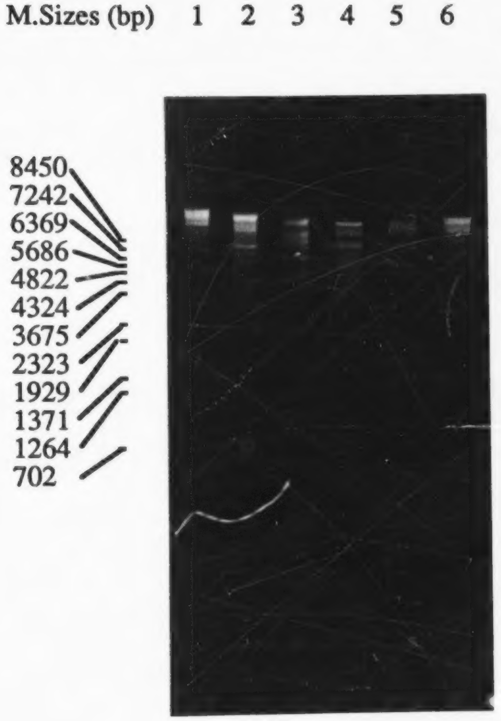

M.Sizes (bp)
1
2
3
4
5
6
8450
7242
6369
5686
4822
4324
3675
2323
1929
1371
1264
702

Fig. 3.1. Conventional agarose gel electrophoresis of restriction endonuclease digests of *M. hyopneumoniae* DNA with *Sca* I (lane 2), *Eco*R V (lane 4), and *Hpa* I (lane 6); and restriction digests of *M. flocculare* DNA with *Sca* I (lane 1), *Eco*R V (lane 3), and *Hpa* I (lane 5). The *Bst*E II digested lambda DNA is indicated at the left as molecular size marker.

To solve this problem, restriction enzymes that recognize G+C rich sequences, were tested to generate smaller numbers of larger restriction fragments. The following restriction enzymes were tested to create relatively larger restriction fragments: *Apa* I, *Asp*718 I, *Bam* H I, *Bgl* I, *Bss* H II, *Bst* E II, *Mlu* I, *Nar* I, *Not* I, *Pst* I, *Pvu* I, *Pvu*II, *Sac* I/*Sst* I, *Sac* II, *Sal* I, *Sma* I, *Sno* I/ *Apa* LI, *Stu* I, *Sty* I, and *Xho* I. After digestion, DNA samples were electrophoresed on 1.0% agarose gels with field-inversion gel electrophoresis (FIGE). DNA samples from *M. flocculare* and *M. hyopneumoniae* were resistant to digestion by some of these enzymes. The digestion results are summarized in Table 3.1. The results obtained after cleavage of *M. flocculare* DNA with *Asp*718 I, *Apa* I, and *Sal* I are shown in Fig. 3.2; and digestion of *M. hyopneumoniae* with *Asp*718 I, *Apa* I, and *Sno* I are shown in Fig. 3.3. Reproducible, complete digestion was achieved with all enzymes, excepting the digestion of *M. flocculare* genomic DNA with *Sal* I. Despite trying several different digestion conditions, the two partial cleavage products (Fig. 3.2, lane 1: two faint bands with sizes of 170 and 130 kbp) still remained. These two partial digestion products were confirmed by double digestion and by hybridization results. The restriction patterns generated by these enzymes readily distinguished these two species. For example, digestion of the intact *M. flocculare* genomic DNA with *Asp*718 I generated seven restriction fragments with sizes ranging from 30-380 kbp while digestion of the intact *M. hyopneumoniae* DNA with the same enzyme produced nine restriction fragments with sizes ranging from 50-170 kbp. In no case were the number and the size of the restriction fragments generated by the endonucleases the same or similar for both mycoplasma species.

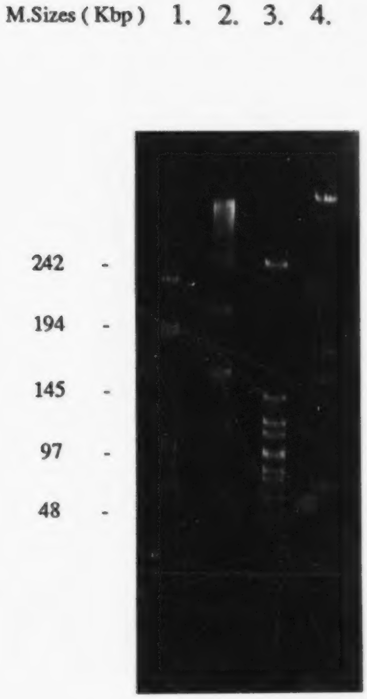

M.Sizes (Kbp)
1.
2.
3.
4.
242 -
194 -
145 -
97 -
48 -

Fig. 3.2. FIGE of restriction digests of the *M. flocculare* chromosome with *Sal* I (lane 1), *Apa* I (lane 3), and *Asp*718 I (lane 4). Lane 2 is a lambda ladder marker. The molecular sizes of the lambda ladder are indicated at the left.

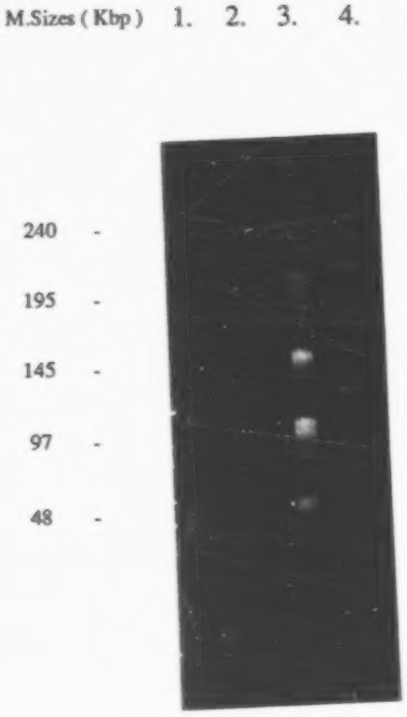

M.Sizes (Kbp)
1.
2.
3.
4.
240 -
195 -
145 -
97 -
48 -

Fig. 3.3. FIGE of restriction digests of the *M. hyopneumoniae* chromosome with *Apa* I (lane 1), *Asp*718 I (lane 2), and *Sno* I (lane 4). Lane 3 is a lambda ladder marker. The molecular sizes of the lambda ladder are indicated at the left.

Table 3.1 Digestion of the intact *M. hyopneumoniae* (Mhp), *M. flocculare* (Mf) and *M. hyorhinis* (Mhr) genomic DNA with rare-cutting restriction endonucleases.

Enzymes	**Mhp**	**Mf**	**Mhr**
Apa I	+	+	-
Apa L I/*Sno* I	+	+	+
*Asp*718 I	+	+	+
Bam H I	+	+	-
Bgl I	+	+	p
Bss H II	+	+	p
Bst E II	p	N.D.	N.D.
Kpn I	p	p	N.D.
Ksp I	N.D.	N.D.	p
Mlu I	+	+	p
Nae I	+	N.D.	-
Not I	+	+	p
Pst I	-	p	+
Puv II	+	+	+
Sac I	N.D.	N.D.	+
Sal I	+	+*	p
Sma I	+	+	p
Sst I	+	+	N.D.
Sst II	N.D.	N.D.	p
Stu I	N.D.	N.D.	p
Sty I	-	-	+
Xho I	+	+	+

+, Digested; -, Not digested; p, Partial digested; N.D., Not done.

* Including two partial digestion fragments, see Fig. 3.2.

3.2. Comparison of the genomic DNA cleavage patterns of *M. hyorhinis* with those of *M. hyopneumoniae* and *M. flocculare.*

M. hyorhinis, like *M. hyopneumoniae* and *M. flocculare*, is a common porcine mycoplasma and comparison of restriction patterns among these porcine mycoplasmas might provide some evidence of genetic relatedness between them. The following restriction endonucleases with recognition sites rich in A+T residues were tested: *Alu* I, *Bgl* II, *Cla* I, *Dra* I, *Eco*R I, *Eco*R V, *Hind* III, *Hpa* I, *Mbo* I, *Sau*3A I, *Sca* I, and *Xba* I. The *M. hyorhinis* DNA samples were sensitive to all of the above enzymes. No distinguishable difference was found between the distribution patterns of the sizes of *M. hyorhinis* DNA fragments and that of the other two porcine mycoplasmas. These results do not support the claim (Bove and Saillard, 1976; Chan and Ross, 1984; Razin, 1985, 1992) that analysis of restriction patterns could provide useful information about genetic relatedness among mycoplasma species.

Restriction endonucleases with recognition sites rich in G+C residues were also tested: *Apa* I, *Asp*718 I, *Bam*H I, *Bgl* I, *Bss* H II, *Bst* E II, *Mlu* I, *Ngo* M I, *Not* I, *Pst* I, *Pvu* I, *Pvu* II, *Sac* I/*Sst* I, *Sac* II, *Sal* I, *Sma* I, *Sno* I/ *Apa* LI, *Stu* I, *Sty* I, and *Xho* I. The results (Table 3.1) showed marked differences between the restriction patterns of *M. hyorhinis* and those of the other two porcine mycoplasma species. Many enzymes which could digest the *M. hyopneumoniae* and *M. flocculare* DNA samples to completion, achieved only partial or no digestion for the *M. hyorhinis* DNA preparations: *e.g.* *Apa* I, *Mlu* I, and *Sal* I. Some enzymes digested the *M. hyorhinis* DNA samples to completion but generated too many smaller DNA fragments with sizes around 50 kbp which were not useful, *e.g.* *Sno* I and *Pst* I. Several different DNA preparations, different DNA concentrations, and different batches of restriction enzymes were tested with the same results. The digestion results are summarized in Table 3.1. Among the enzymes mentioned above, *Asp*718 I was the only one which could digest the *M. hyorhinis* DNA samples to completion and generate reasonable numbers of large DNA fragments. Of the nine *Asp*718 I bands seen on the FIGE gels (data not shown), *Asp* hr1 was much stronger than all other bands suggesting that *Asp* hr1 might contain two fragments of approximately equal sizes. However, no other data could be obtained to verify this assumption. The resistance to digestion by these enzymes could distinguish *M. hyorhinis* from the other two porcine mycoplasma species.

3.3. Hybridization of the rRNA probes with chromosomal DNA preparations from *M. flocculare* and *M. hyopneumoniae*.

As mentioned previously, the mycoplasma genomes are rich in A+T residues (Razin 1985) and the three rRNA genes in most of the mollicutes are close to each other, with only a few exceptions (Amikam *et al.*, 1982, 1984; Chen and Finch, 1989; Frydenberg and Christiansen, 1985; Glaser *et al.*, 1984, 1992; Gobel *et al.*, 1984; Iwami *et al.*, 1984; Neimark, 1983; Razin, 1985; Razin *et al.*, 1983, 1984; Sawada *et al.*, 1981, 1984; Taschke *et al.*, 1986). These enzymes (p. 52) digest the mycoplasma genomic DNA molecules to small pieces and provide more chances to separate these closely linked rRNA genes and therefore provide more information about the mycoplasma rRNA gene organization and the phylogenetic relationship among mycoplasma species.

Based upon these considerations, genomic DNA samples isolated from *M. hyopneumoniae* and *M. flocculare* were digested with selected enzymes (p.52) to produce small fragments. These DNA fragments were size fractionated on 0.8% agarose gels by conventional agarose gel electrophoresis. After transferring the fractionated DNA fragments to nitrocellulose membranes, the membranes were hybridized with different rRNA probes under different stringency conditions to detect fragments with corresponding rRNA genes.

Fig.3.4 is the radioautograph which resulted from probing the Southern transfer membrane of restriction endonucleases digested *M. hyopneumoniae* and *M. flocculare* genomic DNA samples with the *M. hyopneumoniae* 5S rRNA gene specific oligonucleotide probe, Mhp5S17mer. A single major band was detected using DNA from each organism in each digestion. Heterogeneity in the size of the DNA fragments, reflecting the different species, is evident. The hybridization results with different rRNA probes are summarized in Table 3.2. The *E. coli* 16S and 23S rRNA probes were partially purified by sucrose gradient sedimentation. In some cases, hybridization of the digested mycoplasma DNA with these two rRNA probes showed multiple bands with different intensities, indicating cross-contamination between them. It was decided that only strong bands should be used to interpret the results. For some restriction enzymes tested, 16S or 23S rRNA probes reacted with more than one fragment. For example, the *E. coli* 16S rRNA probe reacted with three *M. hyopneumoniae Hpa* I fragments, size 4400 bp, 2700 bp, 1050 bp, while the 23S rRNA probes reacted with only one of them, size 4400 bp. These data indicated that two *Hpa* I restriction sites were located in the 16S rRNA coding region (about 1600 bp) and the 23S rRNA gene and part of the 16S rRNA gene were located on the 4400 bp *Hpa* I fragment. Combined with the results from other enzymes, it was clear that *M. flocculare* , like *M. hyopneumoniae,* had a single copy of rRNA genes (16S, 23S and 5S). In several cases, the 16S and 23S rRNA probes reacted with the fragment with same sizes which suggested that both genes or portions thereof were present in that fragment. The small size of some of these fragments, *e.g.* 4400 bp *M. hyopneumoniae Hpa* I fragment, makes it unlikely to accommodate multiple copies of 16S or 23S rRNA genes. In addition, the small size of these mycoplasma DNA fragments, *e.g.* 4400 bp *M. hyopneumoniae Hpa* I fragment and 4850 bp *M. flocculare Bgl* II fragment, indicated close linkage between the 16S and 23S rRNA genes in these two porcine mycoplasma species. In some cases the size of the fragments which accommodated both the 16S and the 23S rRNA genes could easily accommodate all three rRNA genes. However, with the enzymes tested (p. 52),

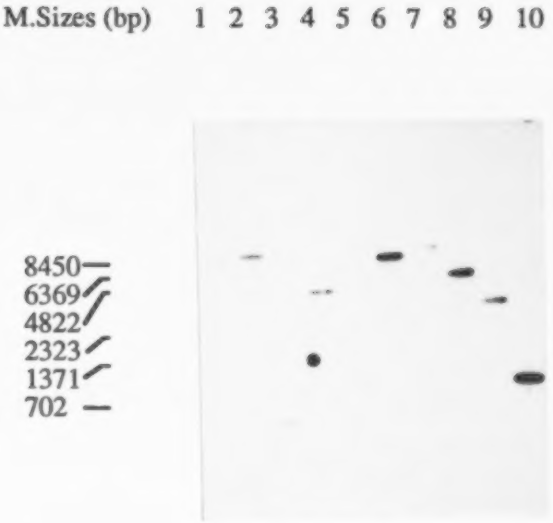


Fig. 3.4. Southern analysis of restriction digests of *M. flocculare* and *M. hyopneumoniae* DNA with *M. hyopneumoniae* 5S rRNA specific oligonucleotide probe, Mhp5S17mer. Restriction digests of *M. flocculare* DNA with *Hpa* I, *Xba* I, *Bgl* II, *Eco*R I, and *Hin*d III are shown in lane 1, lane 3, lane 5, lane 7, and lane 9 respectively. Restriction digests of *M. hyopneumoniae* DNA with *Hpa* I, *Xba* I, *Bgl* II, *Eco*R I, and *Hin*d III are shown in lane 2, lane 4, lane 6, lane 8, and lane 10 respectively. The BstE II digested lambda DNA fragments are indicated at the left as molecule size markers.

Table 3.2. Hybridization data of restriction fragments from *M. hyopneumoniae* (Mhp), *M. flocculare* (Mf), and *M. hyorhinis* (Mhr) probed with the *E. coli* 16S, 23S, and 5S rRNA probes. The sizes (bp) of the positive bands are indicated in this table.

Probe	*Eco*R I*	*Eco*R V*	*Xba* I*	*Hin*dIII*	*Hpa* I*	*Bgl* II*	*Cla* I*	*Sca* I*
Mhp 23S	7050	10000	11000	2500	4400 2700 1050	3200	10000	11000
Mhp 16S	7100 1410	5500	11000	1600	4400	2550	10000	11000
Mhp 5S	7700	2150	5250	1150	11000	10000		10000
Mf 23S	6900	6300	10000 1550	3700 2500	10000 1600	4850	10000	12000
Mf 16S	7000	10000 6400	10000	1600	10000	4850	10000	11000
Mf 5S	12000		700	4200	12000	12000	9500	850
Mhr 23S	9000	12000	10000	1100	8000			11000
Mhr 16S	9000 6500	12000	10000 5300	1500	8000 2500 350			11000
Mhr 5S	7550	4000	3700	2300	6750			6900

*Restriction endonucleases used in this study.

no fragment that reacted with the 5S rRNA probes reacted with either the 23S or 16S rRNA probes. These data provided clear evidence that the *M. flocculare* 5S rRNA gene, like the *M. hyopneumoniae* 5S rRNA gene, was separated from the closely linked 16S and 23S rRNA genes. This work was done early in the study, using partially purified 16S and 23S *E. coli* rRNA probes, when the cloned 16S and 23S rRNA probes were not available. These partially purified probes adequately served the purpose of determining the number of the *M. flocculare* rRNA genes. Later, a *M. hyopneumoniae* 16S rRNA specific oligonucleotide probe, Mhp16S19mer, was used to verify these early results. The 16S oligo probe reacted with a 7100 bp *Eco* R I fragment from *M. hyopneumoniae* and a 7000 bp *Eco* R I fragment from *M. flocculare*. The hybridization results were also confirmed indirectly in later studies when other enzymes (p. 54) were used to construct the restriction maps for *M. flocculare* and *M. hyopneumoniae*. I decided that there was no need to repeat these early experiments with cloned 16S or 23S rRNA gene probes.

Later, intact genomic DNA samples from the two mycoplasma species were digested with endonucleases that recognize G+C rich sequences to generate relatively large restriction fragments which were then size fractionated on 1.0% agarose gels with FIGE or CHEF. The hybridization data obtained from probing these large DNA fragments with the rRNA probes will be presented later in section 3.6. The results confirmed the close linkage between the 16S and 23S rRNA genes for both organisms and the separation of the 5S rRNA genes from the other two rRNA genes by distances of at least 100 kbp.

3.4. Hybridization of the rRNA probes with *M. hyorhinis* chromosomal DNA.

Genomic DNA samples isolated from *M. hyorhinis* were digested with various restriction enzymes with recognition sites rich in A+T residues. The DNA fragments were fractionated and transferred to nitrocellulose filters, then hybridized with the different rRNA probes under different conditions of stringency. The sizes of DNA fragments reacting with the different rRNA probes are summarized in Table 3.2. For all of the enzymes tested, only one major band was detected by the 5S and 23S rRNA probes. In the case of 16S rRNA probes, two major bands were detected for digests by some enzymes. For example, the 16S rRNA probes reacted with two *M. hyorhinis Hpa* I fragments, size 8000 bp and 2500 bp. One of the two bands (8000 bp) that was positive with 16S rRNA probes reacted with 23S rRNA probes as well. As in the cases of *M. hyopneumoniae* and *M. flocculare* , none of the bands reacting with the 5S rRNA probes reacted with 16S or 23S rRNA probes even though in some cases the sizes of the fragments which accommodated both the 16S and the 23S genes could easily accommodate all three rRNA genes. The results indicated that *M. hyorhinis*, like *M. hyopneumoniae* and *M. flocculare,* had a single copy of each rRNA gene (16S, 23S, 5S) and that its 5S rRNA gene might be separated from the closely linked 16S-23S rRNA gene cluster.

3.5. Sizes of the *M. hyopneumoniae* , *M. flocculare*, and *M. hyorhinis* genomes.

The mycoplasma genomes were digested with restriction enzymes expected to only rarely cut the genomic DNA. The DNA fragments were separated by FIGE or CHEF. The sizes of individual fragments were added to obtain the total genome size. Since both organisms have a low G+C content of 33 mol % (Stemke *et al.*, 1985), the following restriction enzymes with recognition sequences rich in C+G residues were tested for their ability to create relatively large restriction fragments: *Apa* I, *Asp*718 I, *Bam* H I, *Bgl* I, *Bss* H II, *Bst* E II, *Mlu* I, *Nar* I, *Not* I, *Pst* I, *Pvu* I, *Pvu* II, *Sac* I/*Sst* I, *Sac* II, *Sal* I, *Sma* I, *Sno* I/ *Apa* LI, *Stu* I, *Sty* I, and *Xho* I. Based upon the number of fragments generated, which should be between 5 and 15 for FIGE analysis, and the reproducibility of the results, *Apa* I, *Asp*718 I, and *Sal* I were chosen for further experiments for *M.*

flocculare, and *Apa* I, *Asp*718 I, and *Sno* I were chosen for further experiments for *M. hyopneumoniae*. The resulting fragments ranged from approximately 400 kbp to 25 kbp.

Figures 3.2 and 3.3 show agarose gels of restriction digests performed on the intact genomic DNA molecules of *M. flocculare* and *M. hyopneumoniae* (FIGE). The sizes of fragments from the digestion of *M. flocculare* DNA with *Apa* I, *Asp*718 I and *Sal* I, and from the digestion of *M. hyopneumoniae* DNA with *Apa* I, *Asp*718 I, and *Sno* I are presented in Table 3.3. The length of all fragments was determined by using a lambda ladder as size marker when the fragments were smaller than 250 kbp and by using a yeast chromosome size marker when the fragments were larger than 250 kbp. The values in Table 3.3 represent average values from several FIGE runs at identical pulse times and are given to the nearest 5 kbp when the values are under 100 kbp and to the nearest 10 kbp when they are above 100 kbp. The size of the mycoplasma genomes were then determined by summing the size of these DNA fragments. The sizes of the *M. flocculare* genome was 915 kbp from *Apa* I fragments, 940 kbp from *Asp*718 I fragments, and 845 kbp from *Sal* I fragments. The size of the *M. hyopneumoniae* genome was 1010 kbp from *Apa* I fragments, 1065 kbp from *Asp*718 I fragments, and 1020 kbp from *Sno* I fragments. The average genome size of *M. flocculare* is 900 kbp and that of *M. hyopneumoniae*, 1030 kbp, close to the genome sizes of 890 and 1070 kbp respectively (single restriction site cut) reported by Robertson *et al.* (1990).

The sizes of fragments from the digestions of intact *M. hyorhinis* genomic DNA with *Asp*718 I, *Sal* I, *Mlu* I, and *Bgl* I are presented in Table 3.4. The values in the table were determined by using the methods described earlier. By adding all *Asp*718 I fragments, the size of the *M. hyorhinis* genome was determined to be 895 kbp, compared to 820 kbp reported by Weisburg *et al.* (1989). Digestion of *M. hyorhinis* genomic DNA with *Sal* I, *Mlu* I, and *Bgl* I showed 1-3 bands with total lengths much less than 820 kbp. In addition, large DNA fragments were found at the top of the gels. Despite trying several different experimental conditions, the results remained the same which suggested that these

Table 3.3. Length and numbering of restriction fragments of the *M. flocculare* (Mf) and *M. hyopneumoniae* (Mhp) chromosomes.

Fragment	Fragment length(kbp)					
	Mf			Mhp		
	Apa I*	*Asp*718 I*	*Sal* I*	*Apa* I*	*Asp*718 I*	*Sno* I*
1	230	380	220	240	170	200**
2	130	160	190	180	150**	120**
3	110	140	170#	160	130	95
4	100	65**	130#	120**	125	70
5	90	60	110**	60**	95	65
6	80	40	90	40	70	50
7	75	30	75	30	65	40
8	60		50		60	
9	40				50	
Total (kbp):	915	940	845	1010	1065	1020

* Restriction enzymes used to cut the mycoplasmas' genomes.

** Bands containing two approximately equal sized fragments (a and b).

# Partial digest products.

Table 3.4 Length and numbering of restriction fragments of the *M. hyorhinis* chromosome.

Fragment No.	Fragment length (kbp)			
	*Asp*718 I*	*Mlu* I*	*Sal* I*	*Bgl* I*
1	180**	180#	210#	220#
2	120		120#	85#
3	100			70#
4	80			
5	70			
6	55			
7	45			
8	40			
9	25			
Total (kbp)	895			

* Restriction enzymes used to cut the mycoplasma genomes.

** Bands containing two approximately equal sized fragments (a and b).

# Partial digest products.

small bands (Table 3.4) might be partial digestion products. Other restriction endonucleases listed on p. 65 were tried. Because it was not possible to find another enzyme which could digest the intact *M. hyorhinis* genomic DNA to generate 5-15 DNA fragments, these preliminary data could not be confirmed.

3.6. Construction of macro-restriction maps of *M. hyopneumoniae* and *M. flocculare* genomes.

The reported values of the total genome sizes (section 3.5) for *M. flocculare* and *M. hyopneumoniae* (Robertson *et al.*, 1990) have been used for the construction of the maps. The restriction fragments obtained by *Apa* I, *Asp*718 I, and *Sal* I digestions of *M. flocculare* DNA and the bands obtained by *Apa* I, *Asp*718 I, and *Sno* I digestions of *M. hyopneumoniae* DNA were numbered as indicated in Table 3.5. Some bands contained more than one fragment. Based upon the intensity of the bands, double digestion, and hybridization data, it was concluded that *Asp* f4, *Sal* f5, *Sno* h1, *Sno* h2, *Apa* h4, *Apa* h5, and *Asp* h2 consisted of two fragments (a and b). Based upon the intensity of the bands and the hybridization data, it was also concluded that *Sal* f3 and *Sal* f4 bands were partial cleavage products.

A standard method has been used to construct the physical maps (Pyle and Finch, 1988). The physical maps are based on hybridization, and single- and double-digestion studies. The primary method for ordering the fragments from a particular digest was to test for hybridization, and therefore overlap, with individual fragments separated from other digests. Membranes containing sets of separated fragments from digestion with several endonucleases were probed with a DNA fragment probe isolated from another gel. Most of the bands, as shown in Fig. 3.2 and 3.3, were isolated from agarose gels and used as probes in Southern analysis. The data from the hybridization experiments are summarized in Table 3.5. Because weak bands can confuse the interpretation of the results, only

Table 3.5 Hybridization data of *M. flocculare* and *M. hyopneumoniae* DNA restriction fragments.

Probe	Fragments positive with the probes
	M. flocculare
Asp f1	*Apa* f2, f4, f5, f6, f7, f8; *Sal* f2, f8*;
Asp f2	*Apa* f1; *Sal* f1, f5, f8*;
Asp f3	*Apa* f1, f2;
Asp f4**	*Apa* f3, f9;
Asp f5	*Apa* f1;
Asp f6	*Apa* f1;
Asp f7	*Apa* f3;
Apa f1	*Asp* f2, f3*, f5*, f6*;
Apa f2	*Asp* f1*, f3, f5*; *Sal* f2; *Sno* f1;
Sal f1	*Asp* f2, f3, f5, f6;
Sal f2	*Apa* f2, f5, f7; *Asp* f1*, f3;
Sal f4#	*Sal* f5, f7; *Asp* f2, f4, f7; *Apa* f1, f3, f6, f9;
Sal f5**	*Asp* f1*, f2*, f4; *Apa* f3, f4;
Sno f1	*Asp* f1, f3; *Apa* f1*, f2, f5*;
*Bam*H f1	*Asp* f1, f4, f7; *Apa* f3, f4;
Bgl f1	*Asp* f4, f7; *Apa* f1*, f3, f9;
M13 5S clone	*Asp* f1; *Apa* f4; *Sal* f3, f5;
pF1-6 (16S+23S)	*Asp* f4, f7; *Apa* f1*, f3, f9*;
	M. hyopneumoniae
Apa h1	*Sno* h2, h3, h4, h6; *Asp* h2;
Apa h2	*Sno* h1; *Asp* h1;
Apa h3	*Sno* h1; *Asp* h1, h3; *Mlu* h1;
Apa h4**	*Sno* h1, h2, h5; *Asp* h2, h3, h5;
Apa h5**	*Sno* h2; *Asp* h1, h2, h3;
Sno h1**	*Apa* h2, h3, h4, h5; *Asp* h1, h3;
Sno h2**	*Apa* h1, h4, h5;
Sno h3	*Apa* h1;
Sno h4	*Apa* h1, h5, h6, h7;
Sno h5	*Apa* h4;
Asp h1	*Apa* h1*, h2, h5; *Sno* h1, h2;
Mlu h1	*Apa* h2, h5; *Sno* h1, h2; *Asp* h1;
Mlu h3	*Apa* h3, h4; *Sno* h1; *Asp* h3;
37N	*Sno* h7; *Apa* h2; *Asp* h4;
50N	*Apa* h4; *Sno* h2; *Asp* h2, h5;
16S+23S rRNA	*Apa* h2; *Sno* h1; *Asp* h4;
Mhp5S17mer	*Apa* h4; *Sno* h2; *Asp* h5;

* Weak signal. ** Probes containing two fragments (see Table 3.3). # Partial digest products.

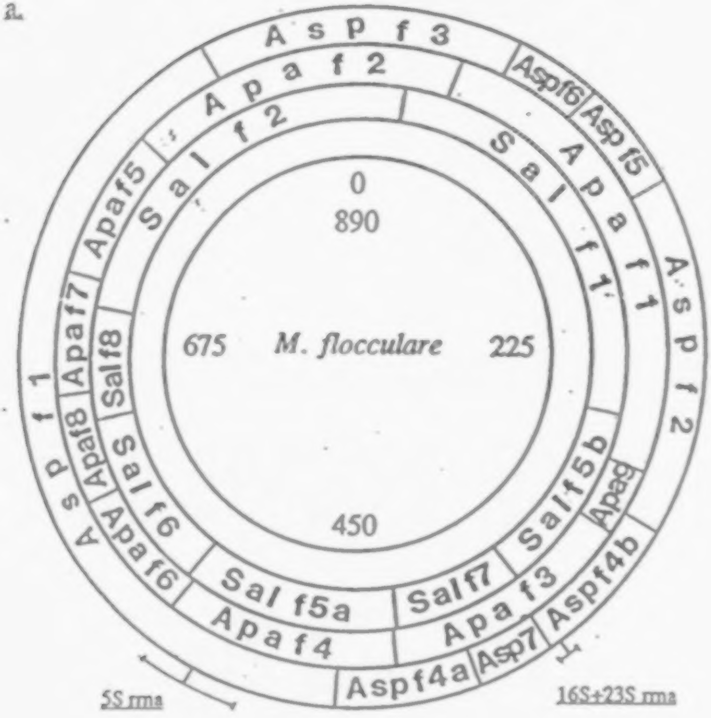
& For fragments symbols see Table 3.3.

strong bands were chosen to construct the maps. In some cases, the weak bands were combined with double digestion results to order the fragments.

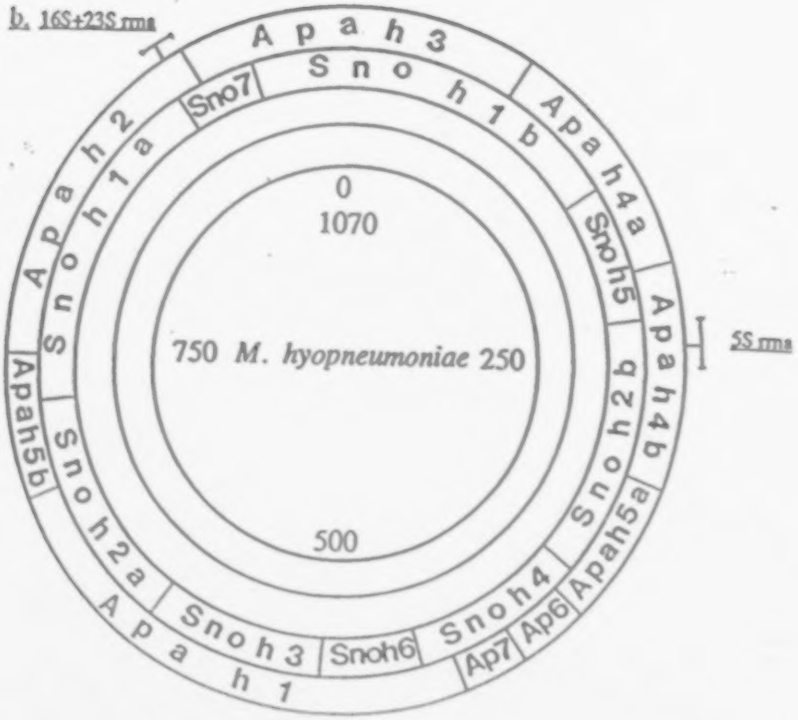
First, the single digest products were ordered to construct the restriction maps. For example, with the *Asp*718 I digestion products of *M. flocculare*, linkage of *Asp* f1 and *Asp* f3 was indicated by hybridization with *Apa* f2 and *Sal* f2; linkage of *Asp* f1 and one of *Asp* f4 fragments (*Asp* f4a) was indicated by hybridization with *Bam*H f1 and *Sal* f4, and so on as indicated in Table 3.5. With the provisional ordering of the fragments from individual digests, integration of these individual maps required an assumption of a standard genome size, since the total of the fragment sizes for various digests differed (Table 3.3). The reported sizes (section 3.5) for *M. flocculare* and *M. hyopneumoniae* (Robertson *et al.*, 1990), have been used as the standard genome sizes. The resulting physical maps are shown in Fig. 3.5a and 3.5b. It was found that *Apa* f1 cross-hybridized to *Asp* f5, but *Apa* f2 also hybridized weakly to *Asp* f5. However, when *Asp* f5 was used as a probe, it only reacted with *Apa* f1 and *Apa* f1-2, a double digestion product from *Apa* f1. I concluded that *Asp* f5 overlapped with *Apa* f1, not with *Apa* f2. Both *Asp* f1 and *Asp* f2 probes reacted weakly with *Sal* f8, but double digestion data showed that *Asp* f1 had four *Sal* I fragments and *Asp* f2 only had two *Sal* I fragments. Based on these data, it was concluded that *Sal* f8 overlapped with *Asp* f1. The relative position of four fragments, *Asp* f5/ *Asp* f6 and *Apa* h6/ *Apa* h7, could not be determined since no appropriate overlapping fragments could be obtained as the probes.

Fig. 3. 5. Macrorestriction maps of the (a) *M. flocculare* ATCC 27716 genome, and (b) *M. hyopneumoniae* ATCC 25095 genome. The middle of *Asp* f3 and *Apa* h3 fragments were arbitrarily placed at 0 kbp for *M. flocculare* and *M. hyopneumoniae*, respectively. For fragment symbols see Table 3.3.

a.


Asp f3
Apa f2
Sal f2
Asp f6
Asp f5
Sal f1
Apa f1
Asp f2
Apa f5
Apa f7
Sal f8
Apa f8
Asp f1
Sal f6
Apa f6
Sal f5b
Apa f9
Sal f5a
Sal f7
Apa f3
Aspf4b
Apa f4
Asp7
Aspf4a
0
890
675
M. flocculare
225
450
5S rrna
16S+23S rrna

b.


16S+23S rrna
Apah3
Sno7
Snoh1b
Apah2
Snoh1a
Apah4a
Snoh5
Apah4b
5S rrna
Snoh2b
Apah5b
Snoh2a
Apah5a
Snoh4
Ap6
Ap7
Snoh3
Snoh6
Apah1
0
1070
750
M. hyopneumoniae
250
500

3.7. Localization of the rRNA genes on the *M. hyopneumoniae* and *M. flocculare* genomes.

Several rRNA probes were used to locate the positions of the rRNA genes on the genome maps and the results are shown in Table 3.5. For neither species did the fragments reacting with 16S or 23S probes react with the 5S probes, providing further evidence that their 5S rRNA genes were separated from the 16S and 23S rRNA genes. The M13 5S probe hybridized to *Asp* f1, *Apa* f4, and *Sal* f5, but not to *Bam* H f1 which overlapped *Asp* f1, f4, f7. The *M. flocculare* 5S rRNA gene should reside in the region common to *Asp* f1, *Apa* f4, and *Sal* f5, excluding the area overlapped with *Bam* H f1. The 5S oligo probe, Mhp5S17mer, hybridized to *Apa* h4, *Sno* h2, and *Asp* h5; the 50N probe reacted to *Apa* h4, *Sno* h2, *Asp* h2, and *Asp* h5; *Apa* h4 and *Apa* h5 cross-hybridized to *Asp* h2. Based upon this information, it was concluded that the *M. hyopneumoniae* 5S rRNA gene was located in a region overlapped *Apa* h4, *Sno* h2, and *Asp* h5, but excluding the area overlapped *Asp* h2. The 37N probe reacted with *Apa* h2, *Asp* h4, and *Sno* h7. The 16S+23S rRNA probe hybridized to *Apa* h2, *Asp* h4, *Sno* h1; in addition, a weak signal was found at the *Sno* h7 position. It was concluded that the *M. hyopneumoniae* 23S and 16S rRNA genes resided in the area overlapped *Apa* h2, *Asp* h4, *Sno* h1, and *Sno* h7. The location of the 5S, 16S and 23S rRNA genes of the two mycoplasmas is indicated in Fig. 3.5a and 3.5b. The distance between the 5S and 16S /23S rRNA genes is at least 150 kbp for *M. flocculare* and about 270 kbp for *M. hyopneumoniae*.

3.8. Comparison of the DNA restriction cleavage patterns of the two "*M. fermentans*" strains.

As indicated in section 2.2, two separated harvests (Mfer-A and Mfer-B) of ATCC strain 19989, proported to be the type strain for *M. fermentans*, and *M. fermentans* (*incognitus* strain) were obtained. In this study, the restriction patterns of Mfer-A DNA were compared with those of *M. fermentans* (*incognitus* strain) DNA. We tested the following restriction enzymes: *Bgl* II, *Dra* I, *Eco*R I, *Eco*R V, *Hin*d III, *Hpa* I, *Sca* I, and *Xba* I. *M. fermentans* DNA samples are sensitive to all of these enzymes and the restriction patterns of the DNA of these two "*M. fermentans*" strains are different with many distinguishable bands.

The intact genomic DNA samples from these two "*M. fermentans* strains" (ATCC19989 and *incognitus* strain) were also tested with the following enzymes: *Apa* I, *Asp*718 I, *Bam*H I, *Bgl* I, *Mlu* I, *Not* I, *Pst* I, Sac I/*Sst* I, *Sac* II, *Sal* I, *Sma* I, *Apa*L I, *Sty* I, and *Xho* I. The digestion results are summarized in Table 3.6. When the DNA fragments generated by these enzymes were separated by FIGE or CHEF, the differences between the cleavage patterns of these two "*M. fermentans* strains" became obvious. The results (CHEF) from the digestions of *M. fermentans* (*incognitus* strain) DNA with *Mlu* I, *Bgl* I, and *Bam*H I are shown in Fig. 3.6. The results (FIGE) from the digestions of *M. fermentans* (*incognitus* strain) DNA with *Bgl* I, *Mlu* I-*Bgl* I (double digest), *Mlu* I, *Mlu* I-*Bam*H I (double digest) and *Bam*H I are shown in Fig. 3.7. For *M. fermentans* (type strain, ATCC 19989), two different batches of mycoplasma cells (Mfer-A and Mfer-B) were used and their DNA restriction patterns are shown in Fig. 3.8 (Mfer-A) and Fig. 3.9 (Mfer-B). It was noted that the cleavage patterns of Mfer-A are quite different from that

Table 3.6 Digestion of the intact Mfer-A and Mfer-B genomic DNA and *M. fermentans* (*incognitus* strain, Mi) genomic DNA with restriction enzymes.

Enzymes	**Mfer-A**	**Mfer-B**	**Mi**
Apa I	-	p	-
Apa L I/*Sno* I	+	+	+
*Asp*718 I	+	-	+
Bam H I	+	p	+
Bgl I	+	+	+
Mlu I	+	+	+
Ngo M I	p	N.D.	p
Not I	-	N.D.	-
Sac II	p	N.D.	p
Sal I	+	+	+
Sma I	p	+	p
Sty I	-	N.D.	N.D.
Xho I	p	-	p

+, Digested; -, Not digested; p, Partial digested; N.D., Not done

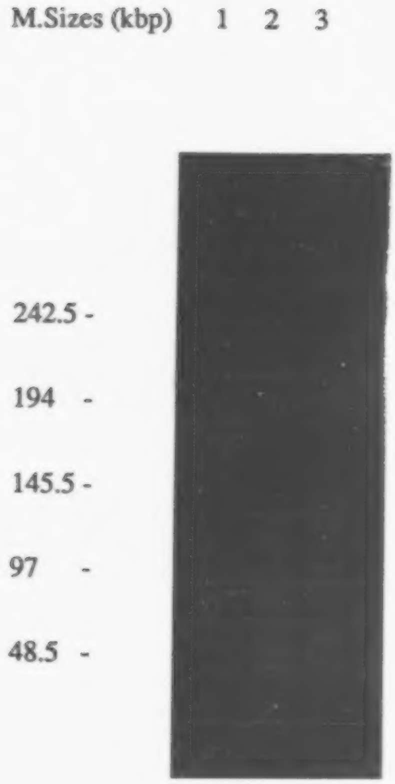

M.Sizes (kbp)
1
2
3
242.5 -
194 -
145.5 -
97 -
48.5 -

Fig. 3.6. CHEF of restriction digests of the intact *M. fermentans* (*incognitus* strain) chromosome with *Mlu* I (lane 1), *Bgl* I (lane 2), and *Bam* H I (lane 3). The sizes of a lambda ladder are indicated at the left as molecular size marker. The ramping time was 5-30 s.

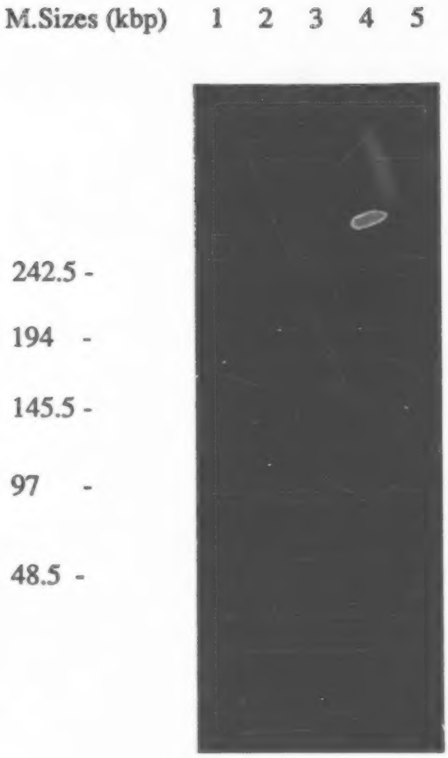

M.Sizes (kbp)
1
2
3
4
5
242.5 -
194 -
145.5 -
97 -
48.5 -

Fig. 3.7. FIGE of restriction digests of the intact *M. fermentans* (*incognitus* strain) chromosome with *Bgl* I (lane 1), *Bgl* I-*Mlu* I (lane 2), *Mlu* I (lane 3), *Mlu* I-*Bam*H I (lane 4), and *Bam*H I (lane 5). The sizes of a lambda ladder are indicated at the left as molecular size marker.

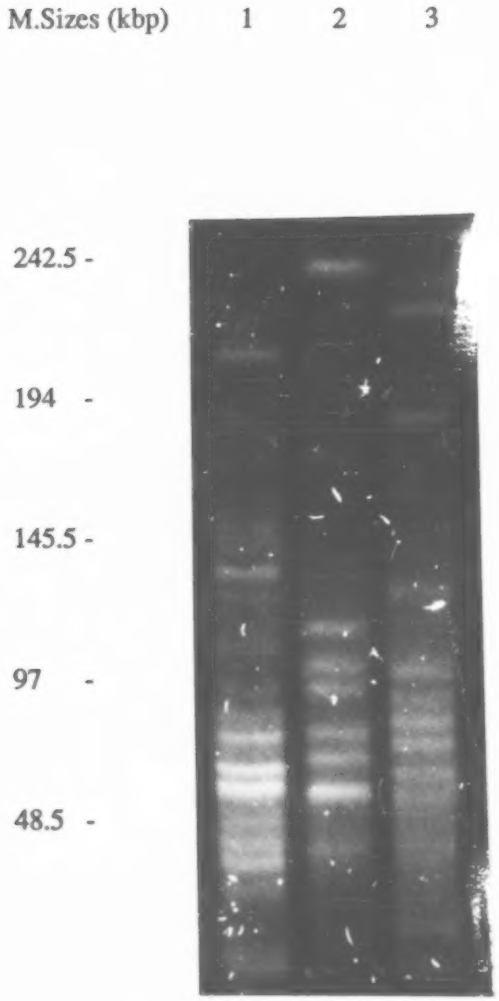

M.Sizes (kbp)
1
2
3
242.5 -
194 -
145.5 -
97 -
48.5 -

Fig. 3.8. CHEF of restriction digests of the intact Mfer-A chromosome with *Sal* I (lane 1), *Mlu* I (lane 2), and *Bgl* I (lane 3). The sizes of a lambda ladder are indicated at the left as molecular size marker. The ramping time for this gel was 5-30 s.

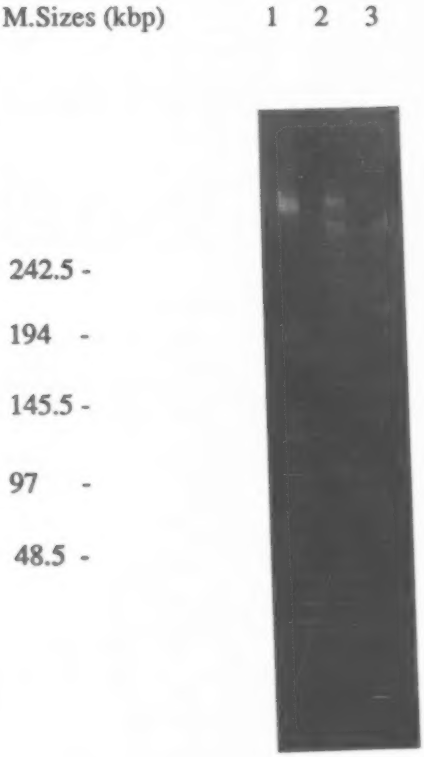

M.Sizes (kbp)
1
2
3
242.5 -
194 -
145.5 -
97 -
48.5 -

Fig. 3.9. FIGE of restriction digests of Mfer-B chromosome with *Mlu* I (lane 1), *Sal* I and *Mlu* I (lane 2), and *Sal* I (lane 3). The sizes of a lambda ladder are indicated at the left as molecular size marker.

of Mfer-B. For example, the restriction digestion of the intact Mfer-A DNA with *Sal* I generated more than 15 fragments with sizes ranging from 200 to 30 kbp while the restriction digestion of the intact Mfer-B DNA with *Sal* I generated only 5 fragments with sizes ranging from 500 to 110 kbp. Similar results were found with other enzymes, *e.g.* *Bam* H I, *Sma* I, and *Asp*718 I. These data combined with other results presented later in this chapter, indicate that, genetically, Mfer-A and Mfer-B are different. These results will be discussed in detail in Chapter IV.

3.9. Hybridization of the rRNA probes with *M. fermentans* (*incognitus* strain) and Mfer-A chromosomal DNA.

Chromosomal DNA samples from *M. fermentans* (*incognitus* strain) and Mfer-A were digested with enzymes (p. 75, line 7) and then hybridized with different rRNA probes to detect the corresponding DNA fragments. Up to five positive bands hybridized with the 16S or 23S rRNA probes while only two bands hybridized with the 5S rRNA probes. The number and sizes of these positive bands were quite different for these two DNA preparations. It is further indication that, genetically, these two organisms differ significantly.

It is apparent that the genomes of these two "*M. fermentans*" strains have more than one set of rRNA genes. However, it was noted that the intensities of these positive bands were different. The strong bands could indicate the presence of multiple fragments because these enzymes generated many DNA fragments with the same or similar sizes. It is difficult to determine the exact number of rRNA genes based upon the hybridization data obtained from these enzymes. To solve this problem, other enzymes (p. 75, line 13-14) were used to cleave intact *M. fermentans* intact genomic DNA molecules into fewer large fragments which were then hybridized with the rRNA probes. The hybridization results of digested *M. fermentans* (*incognitus* strain) DNA with different rRNA probes will be presented later in section 3.12. The results show that the genome of *M. fermentans* (*incognitus* strain) contains two sets of rRNA genes (16S, 23S, 5S). However, *M. fermentans* (*incognitus* strain) has a quite unusual rRNA organization: it contained four separated rRNA operons and both of the 5S rRNA genes were separated from the two closely linked 16S-23S rRNA gene clusters (see section 3.12). Preliminary data indicated that Mfer-A had two 16S rRNA genes while the results for 23S and 5S rRNA genes have not yet been determined. Preliminary results from another *M. fermentans* (type strain, Mfer-B) showed that its genome contained two sets of rRNA genes (16S, 23S, 5S). These data also suggest that these two organisms were quite different.

3.10. The genome sizes of "*M. fermentans*" strains.

To determine the genome sizes of *M. fermentans* strains, the intact mycoplasma genomic DNA samples were digested with restriction enzymes and run on FIGE or CHEF gels to separate the DNA fragments. The total genome sizes were obtained by adding the size of the individual fragments. The following restriction enzymes with recognition sequences rich in G+C residues were used to generate large DNA fragments: *Apa* I, *Asp*718 I, *Bam*H I, *Bgl* I, *Mlu* I, *Not* I, *Pst* I, *Pvu* I, *Pvu* II, *Sac* I/*Sst* I, *Sac* II, *Sal* I, *Sma* I, *Sno* I/*Apa*L I, *Sty* I, and *Xho* I. Based upon the number of DNA fragments generated and the reproducibility of the results, *Mlu* I, *Bam*H I, and *Bgl* I were chosen for further experiments for *M. fermentans* (*incognitus* strain). For *M. fermentans* (type strain) the DNA from two batches of cells (Mfer-A and Mfer-B) showed different restriction patterns. *Mlu* I and *Bgl* I were chosen for Mfer-A while *Mlu* I and *Sal* I were chosen for Mfer-B.

Fig. 3.6 shows a CHEF gel of restriction digestion of the intact genomic DNA of *M. fermentans* (*incognitus* strain). An exponentially increasing forward switch time from 5 to 30 s to separate DNA fragments ranging from 20 to 250 kbp. When the sizes of DNA fragments were larger than 250 kbp, exponentially increasing forward switch time from 15 to 60 s were used to separate these large fragments (CHEF). The sizes of fragments from the digestion of *M. fermentans* (*incognitus* strain) with *Mlu* I, *Bgl* I, and *Bam* H I are presented in Table 3.7. The length of all fragments was determined using a lambda ladder as a size marker when the fragment size was smaller than 250 kbp (FIGE or CHEF). When the fragment size was larger than 250 kbp, a yeast chromosome ladder was applied as a size marker (CHEF). Under the experimental conditions used in this study, *Mlu* I1 (590 kbp) often showed two weak bands with a smear between them at the top of the lane on FIGE gels (Fig. 3.7). The results from CHEF gels and hybridization analysis indicated that the smear represented only one DNA fragment. The values shown in Table 3.7 represent the average values from several FIGE or CHEF runs and are given to the nearest 5 kbp when the value is under 100 kbp and to the nearest 10 kbp when it is above 100 kbp. The sizes of the mycoplasma genomes were then determined by adding all of these DNA fragments. The genome size of *M. fermentans* (*incognitus* strain) was 965 kbp from *Bgl* I fragments, 1019 kbp from *Mlu* I fragments, and 958 kbp from *Bam* H I fragments. The average genome size of *M. fermentans* (*incognitus* strain) was 980 kbp, which was significantly smaller than the genome size of 1160 kbp reported by Neimark and Lange (1990) for another strain of *M. fermentans* (GII strain).

The results obtained after cleavage of intact genomic DNA from Mfer-A with *Mlu* I and *Bgl* I are shown in Fig. 3.8 (CHEF); cleavage patterns of genomic DNA from Mfer-B with *Mlu* I, *Sal* I and *Mlu* I-*Sal* I (double digestion) are shown in Fig. 3.9 (FIGE). The sizes of the DNA fragments generated by these enzymes are summarized in Table 3.8. The sizes of the mycoplasma genomes were determined by adding all of these fragment sizes. According to the intensity of the bands, it was concluded that *Mlu* fa 4, fa 8, and *Bgl* fa 4 contained two fragments. Further experiments have not been performed to confirm these preliminary results. In addition, some of the bands are weaker than other bands close to them

Table 3.7 Length and enumeration of restriction fragments of the intact *M. fermentans* (*incognitus* strain) chromosome.

Fragment No.	Fragment length (kbp)		
	Bgl I*	*Mlu* I*	*Bam* H I*
1	230	590	190
2	160	160	130
3	110**	100	110**
4	100	80	90
5	65**	70	80
6	50	15	70
7	40	5	50**
8	30		35**
9			15
Total	960	1020	965

* Restriction enzymes used to cut the mycoplasma genomes.

** Bands containing two approximately equal sized fragments (a and b).

Table 3.8 Length (kbp) and numbering of the restriction fragments of the Mfer-A and Mfer-B chromosomes.

Fragment No.	Mfer- A		Mfer-B		
	Mlu I*	*Bgl* I*	*Mlu* I*	*Sal* I*	*Mlu* I-*Sal* I*
1	230	220	500	500#	500
2	170	180	330	300	300
3	120	130	190	210	120
4	100**	100**	120	160	100
5	95	90	60	110	75
6	80	80	45		70
7	70	75	30		55
8	60**	70			40
9	40	60			
10		40			
11		10			
Total (kbp)	1125	1155	1275	1280	1260

* Restriction enzymes used to cut the mycoplasma genomes.

** Bands containing two approximately equal sized fragments (a and b).

# Obtained from double digestion data.

which could be an indication of partial digestion. More study needs to be done to confirm the identity of Mfer-A and Mfer-B before any meaningful interpretation of these preliminary data can be achieved.

3.11. Construction of macro-restriction map of the *M. fermentans* (*incognitus* strain) genome.

The total average genome size (section 3.10) of *M. fermentans* (*incognitus* strain) was used for the construction of the macro-restriction map. The restriction bands obtained by *Mlu* I, *Bgl* I, and *Bam* H I digestions of *M. fermentans* (*incognitus* strain) genomic DNA were numbered as indicated in Table 3.7. Based upon the intensity of the bands, double digestion results, and hybridization data, it was determined that *Bgl* I3, *Bgl* I5, *Bam* I3, *Bam* I 7, and *Bam* I8 consisted of two fragments (a and b) each.

The same method used for construction of physical maps of *M. hyopneumoniae* and *M. flocculare* (section 3.6) was used here to construct a restriction map for *M. fermentans* (*incognitus* strain). The physical map was based on hybridization, single cleavage, and double cleavage studies. For the double digestion studies, instead of cutting out the bands from single digests and digesting with the second enzyme, the intact genomic DNA molecules were digested with two enzymes and the fragments generated were separated on FIGE or CHEF gels. The restriction digestion and the hybridization results from single- and double-digests were then compared with each other. In this way the bands containing two fragments could be identified. For example, probe *Bgl* I3 (110 kbp) hybridized with three *Mlu* I-*Bgl* I double digest products (110, 75, and 30 kbp) which is an indication that *Bgl* I3 consisted of two fragments (a and b) and that one of them contained a *Mlu* I restriction site.

To construct the restriction map, the single digest products were ordered as the first step. For example, with the *Mlu* I digestion products of the organism, linkage of *Mlu* I1 and *Mlu* I2 was indicated by hybridization with *Bgl* I2 and *Bam* I2; linkage of *Mlu* I3 and *Mlu* I5 was indicated by hybridization with *Bam* I3 and *Bgl* I7 (Fig. 3.10). For the *Bam* H I digestion products of the organism, linkage of *Bam* I1, I3, and I8 was indicated by hybridization with *Bgl* I1 (Fig. 3.11), and so on as indicated in Table 3.9. With the provisional ordering of the fragments from individual digests, integration of these individual maps requires an assumption of a standard genome size. An average size (section 3.10) for *M. fermentans* (*incognitus* strain) was used as the standard genome size. The resulting physical map is shown in Fig. 3.12. The hybridization results from probing the *Bam* H I digested mycoplasma DNA with *Mlu* I3 showed two strong bands (*Bam* I3 and *Bam* I5) and a weak band (*Bam* I1). The *Bam* I1 probe reacted strongly with *Mlu* I1 and reacted weakly with *Mlu* I3. The results from other experiments indicated that *Bam* I1 should not overlap with *Mlu* I3. Since both fragments contained the 5S rRNA genes, these weak bands could be caused by cross hybridization between the small 5S rRNA genes on these two fragments. Double digestion results also indicated that *Bam* I1 overlapped with *Mlu* I1. Double digestion of the intact Mi genomic DNA with *Mlu* I and *Bam* H I generated a fragment which had the same size as *Bam* I1 (190 kbp). Since all of the fragments generated by *Mlu* I digestion were smaller than 160 kbp except *Mlu* I1 for 590 kbp (Table 3.7), this 190 kbp fragment must be *Bam* I1 and it should reside inside *Mlu* I1. A similar situation was found in the case of *Bgl* I1.

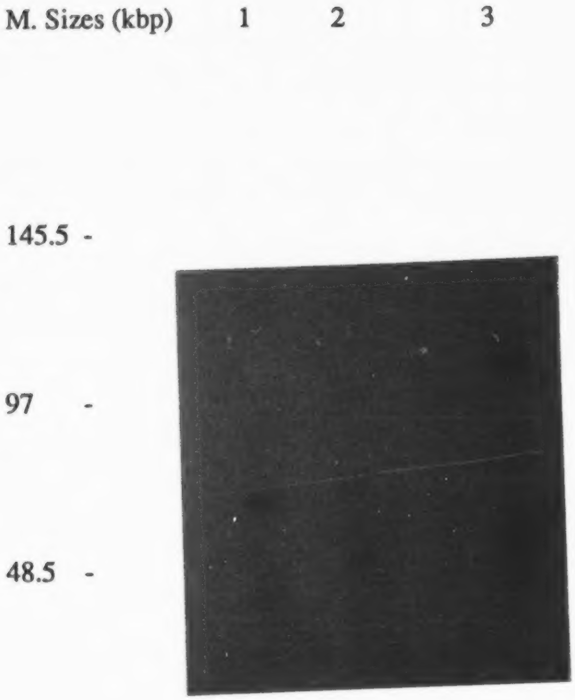


Fig. 3.10. Southern analysis from FIGE of restriction digests of the intact *M. fermentans* (*incognitus* strain) chromosome with *Mlu* I (lane 1), *Bgl* I (lane 2), and *Bam* H I (lane 3) with Mlu I5 probe. The sizes of a lambda ladder are indicated at the left as molecular size marker.

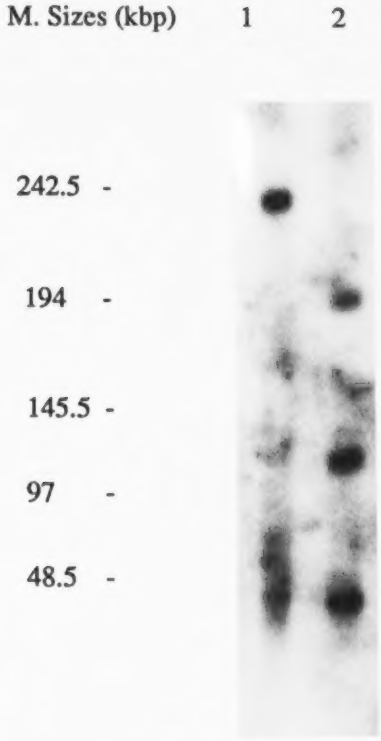

M. Sizes (kbp)
1
2
242.5 -
194 -
145.5 -
97 -
48.5 -

Fig. 3.11. Southern analysis from FIGE of restriction digests of the intact *M. fermentans* (*incognitus* strain) chromosome DNA with *Bgl* I (lane 1) and *Bam* H I (lane 2) with *Bgl* I1 probe. The sizes of a lambda ladder are indicated at the left as molecular size marker.

Table 3.9 Hybridization data of *M. fermentans* (*incognitus* strain) DNA restriction fragments. For fragment symbols see Table 3.6.

Probe	Fragments positive with the probes
Mlu I2	*Bgl* I2, I4, I8; *Bam* I2, I7, I8;
Mlu I3	*Bam* I3, I5; *Bgl* I3, I7*;
Mlu I4	*Bgl* I5; *Bam* I4;
Mlu I5	*Bgl* I5, I7; *Bam* I3;
Bgl I1	*Bam* I1, I3, I8; *Mlu* I1;
Bgl I2	*Bam* I2, I6; *Mlu* I1, I2;
Bgl I3	*Bam* I1, I3*, I5, I7; *Mlu* I1, I3;
Bgl I4	*Mlu* I2; *Bam* I7, I8;
Bam I1	*Bgl* I1, I3, I6; *Mlu* I1;
Bam I2	*Mlu* I1, I2;
Bam I4	*Mlu* I4, I6*;
Sac I2	*Bgl* I2, I4, I8; *Bam* I2, I7, I8;
Sal I1	*Bam* I2, I6;
Sal I3	*Bgl* I2, I8; *Bam* I2, I8;
Mhp5S17mer	*Mlu* I1, I3; *Bgl* I3, I6; *Bam* I1, I5;
p133(16S)	*Mlu* I4, I5, I6, I7; *Bam* I3, I4, I9; *Bgl* I5;
GWS60(23S)	*Mlu* I6, I7; *Bam* I4, I9; *Bgl* I5;

* Weak signal.

** Probes containing two fragments (see Table 3.6).

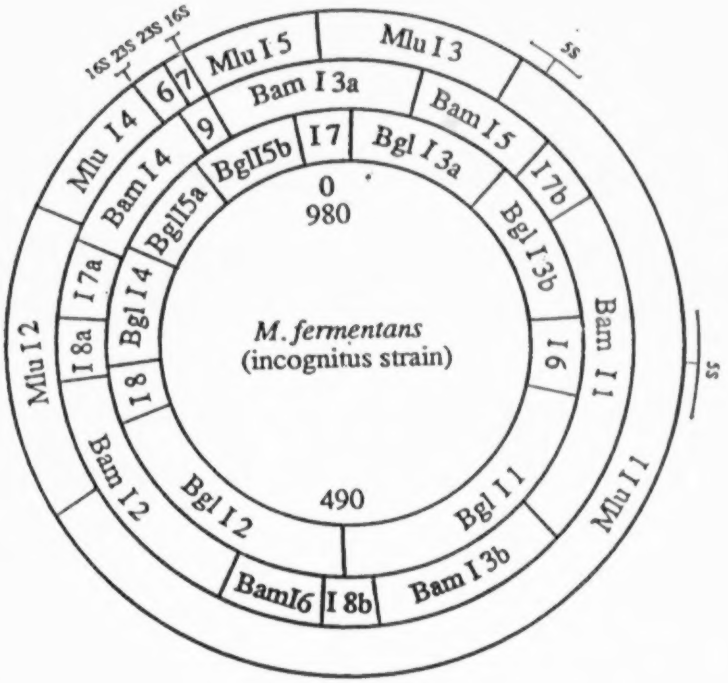

16S 23S 23S 16S
5S
5S
Mlu I 5
Mlu I 3
Mlu I 4
6
7
9
Bam I 3a
Bam I 5
Bam I 4
BglI5b
I 7
Bgl I 3a
I 7b
BglI5a
Bgl I 3b
0
980
Mlu I 2
I 7a
I 8a
Bgl I 4
I 8
M. fermentans
(incognitus strain)
I 6
Bam I 1
Bam I 2
Bgl I 2
490
Bgl I 1
Mlu I 1
BamI6
I 8b
Bam I 3b

Fig. 3.12. Macrorestriction map of the *M. fermentans* (*incognitus* strain) genome. The joining point between the *Mlu* I3 and *Mlu* I5 fragments was arbitrarily placed at 0 kbp. For fragment symbols, see Table 3.6.

3.12. Localization of the rRNA genes on the *M. fermentans* (*incognitus* strain) genome.

Several specific rRNA probes were used to locate the positions of the rRNA genes on the mycoplasma genome map. The hybridization results are shown in Table 3.9. The cloned 16S rRNA probe (p133) reacted with four *Mlu* I fragments (*Mlu* I4, I5, I6, I7), three *Bam* H I fragments (*Bam* I3, I4, I9), and one *Bgl* I fragment (*Bgl* I5, Table 3.9). When the same probe was used to hybridize with the *Mlu* I-*Bgl* I double digested *M. fermentans* DNA, four positive bands (70, 60, 15, 5 kbp) were found. This was direct evidence that *Bgl* I5 contains two fragments which were located side by side on the genome map (Fig. 3.12). The data also indicated that there should be a *Mlu* I restriction site in the 16S rRNA coding region. The *M. fermentans* 16S rRNA gene sequence from GenBank (accession No.M24289) showed two *Mlu* I sites which confirmed this conclusion. The small fragments (332 bp) from *Mlu* I digestion were not detected on FIGE or CHEF gels. The 16S sequence from GenBank also showed one *Bam*H I site, and no Bgl I site. The *M. flocculare* 23S rRNA specific oligonucleotide probe, GWS 60, hybridized to *Mlu* I6, I7, *Bam* I4, I9, and *Bgl* I5 (Table 3.9). These results suggested close linkage between 16S and 23S rRNA genes. Fig. 3.13 is a radioautograph resulting from probing the Southern transfer membrane of restriction digested *M. fermentans* (*incognitus* strain) DNA with *M. hyopneumoniae* 5S rRNA specific oligonucleotide probe, Mhp5S17mer. In lane 3 (*Mlu* I digestion products), the 5S oligo probe hybridized to a strong band at the bottom (*Mlu* I3) and two weaker bands on the top of the lane which, as suggested earlier in this chapter, represent only a single fragment (*Mlu* I1). The 5S probe reacted with *Bam* I1, I5, and *Bgl* I3, I6 as well (see Table 3.9). No DNA fragments reacting with the 5S rRNA probe reacted with the 16S or 23S rRNA probes. These data provided evidence that both 5S rRNA genes of the organism were separated from the closely linked 16S and 23S rRNA gene clusters. The results from the double digestions support this assumption. The

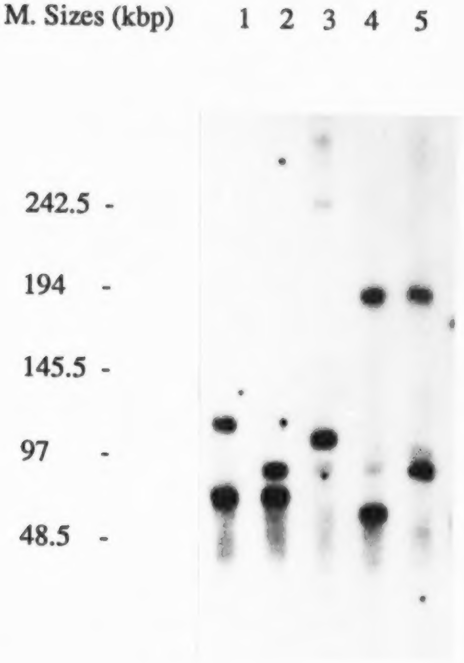


Fig. 3.13. Southern analysis of FIGE of restriction digests of *M. fermentans* (*incognitus* strain) chromosome with *Bgl* I (lane 1), *Bgl* I-*Mlu* I (lane 2), *Mlu* I (lane 3), *Mlu* I-*Bam*H I (lane 4), and *Bam*H I (lane 5) with the *M. flocculare* 5S rRNA specific oligonucleotide probe, Mhp5S17mer. The sizes of a lambda ladder are indicated at left as molecular size marker.

locations of the 5S, 16S, and 23S rRNA genes of this organism have been indicated in Fig. 3.12.

3.13. Construction of a preliminary macrorestriction map of Mfer-B.

The total average genome size of Mfer-B of 1270 kbp was used for the construction of its macro-restriction map. The restriction bands obtained by digesting the mycoplasma DNA with *Mlu* I, *Sal* I, and *Mlu* I-*Sal* I (double digestion) were numbered as indicated in Table 3.8.

The same method for construction of physical maps described earlier in this chapter (section 3.6) was used here. For double digestion studies, instead of cutting out the bands from single digests and digesting with the second enzyme, intact genomic DNA molecules were digested with two enzymes and the fragments generated were separated on FIGE or CHEF gels.

To construct the restriction map, the single digest products were ordered. For example, with the *Mlu* I digestion products of the organism, linkage of *Mlu* fb1 and *Mlu* fb2 was indicated by hybridization with *Sma* fb2; linkage of *Mlu* fb2 and *Mlu* fb7 was indicated by hybridization with *Apa* L fb2; and so on as indicated in Table 3.10. With the provisional ordering of the fragments from individual digests, the average size for Mfer-B was used to integrate these individual maps. The resulting preliminary physical map is shown in Fig. 3.14. The rRNA genes were also located on the map, but I have not been able to determine the distance between 5S and other rRNA genes. It was noted that DNA fragments from Mfer-B did not hybridize with DNA from *M. fermentans* (*incognitus* strain) under the high stringency conditions (section 2.18) used in this study. Under the same conditions, however, the Mfer-A DNA fragments hybridized with DNA from both *M. fermentans* (*incognitus* strain) and Mfer-B. These results will be discussed in chapter IV.

Table 3.10 Hybridization data of Mfer-B DNA restriction fragments.

Probe	Fragments positive with the probes
Sal fb5	*Mlu* fb3; *Sma* fb7;
Sal fb3	*Mlu* fb3, fb6, fb7; *Sma* fb4;
Sal fb4	*Mlu* fb4*, fb5; *Sma* fb3;
Apa L fb2	*Mlu* fb2, fb7; *Sal* fb2, fb3;
Sma fb2	*Mlu* fb1, fb2; *Sal* -*Mlu* fb1
Sma fb5**	*Mlu* fb2, fb3; *Sal* fb5;
Sma fb6	*Mlu* fb3; *Sal* fb3, fb5;
Mlu fb4	*Sal* fb4; *Sma* fb3;
Mlu fb5	*Sal* fb4
p133 (16S)	*Sal* fb2, fb3; *Sma* fb4, fb6;
Mhp5s17mer (5S)	*Mlu* fb2, fb3; *Sal* fb2, fb5; *Sma* fb5;

* Weak signal.

** Probe containing two fragments.

& For fragments symbols see Table 3.7. *Mlu* fb1: Fragment No.1 from restriction enzyme *Mlu* I digestion.

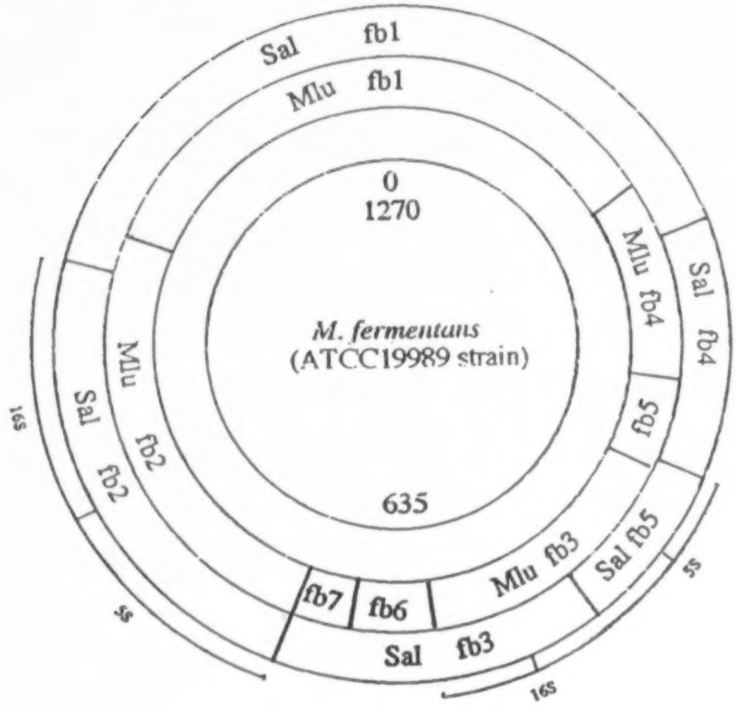

Sal fb1
Mlu fb1
0
1270
M. fermentans
(ATCC19989 strain)
635
Mlu fb4
Sal fb4
fb5
Mlu fb2
Sal fb2
16S
5S
fb7
fb6
Mlu fb3
Sal fb5
Sal fb3
5S
16S

Fig. 3.14. Macrorestriction map of the Mfer-B genome. The middle of the fragment *Mlu* f1 was arbitrarily placed at 0 kbp. For fragment symbols see Table 3.7.

3.14. Cloning of the *M. flocculare* 5S rRNA gene.

One objective of this study was to determine the nucleotide sequence of the *M. flocculare* 5S rRNA gene and to study the transcription regulatory elements of this gene. The first step was to clone the *M. flocculare* 5S rRNA gene, with its upstream and downstream regions, into an *E. coli* host/vector system. Hybridization data showed that the *M. flocculare* 5S rRNA gene was located on a 700 bp *Xba* I fragment (Table 3.2). Since the sizes of the known mycoplasma 5S rRNA are around 107 bp (table 4.1), this 700 bp *Xba* I fragment was considered likely to have the complete 5S rRNA gene with some upstream and downstream regions. Most of the DNA fragments generated by *Xba* I were larger than 3 kbp and the 700 bp fragment was one of the few fragments smaller than 1 kbp which makes cloning of this fragment into a sequencing vector, such as M13 mp 19, favorable. The small size of the insertion would also be likely to stabilize the recombinant M13 phage. The only apparent drawback was the possibility of multi-insertion of the *Xba* I fragments into one sequencing vector. This problem could be solved by restriction mapping of the insertion. After digesting *M. flocculare* DNA with the restriction enzyme *Xba* I, the resulting fragments were ligated into *Xba* I digested M13 mp19 vectors. The recombinant DNA molecules were then transformed into *E. coli* JM 101 competent cells. The ^{32}P labeled *M. hyopneumoniae* 5S rRNA specific oligonucleotide probe, Mhp5S17mer, was used to screen the *M. flocculare* *Xba* I library. Several apparently positive M13 recombinant clones were chosen for further analysis. After amplifying and replating of those recombinant clones, one clone was confirmed as positive and used for subsequent studies.

3.15. Sequencing strategies.

Both single-stranded sequencing and double-stranded sequencing were tried in this study. Double-stranded sequencing using T7 DNA polymerase (from Pharmacia) gave clearer and more reliable results than did single-stranded sequencing using the Klenow fragment of *E. coli* DNA polymerase I (from BMC). The primers used initially were the M13 universal primer, the M13 reverse sequence primer, and a *M. hyopneumoniae* 5S rRNA specific primer, Mhp5S17mer. The resulting sequences were then used to prepare other primers, such as YH3, YH4, and GWS 7 (see Table 2.2), for further sequencing. DNA bands beyond the runs of 18 "T" residues were not clear on the sequencing gels. To solve this problem, one strand was sequenced up to the runs of "T" residues and then the resulting sequences were used to prepare the primer (YH4) immediately after the runs of "T" residues for sequencing the opposite strand. After analysis of the sequences it was concluded that the recombinant M13 clone had a single 665 bp *M. flocculare Xba* I fragment insertion.

3.16. Sequence of the *M. flocculare* 5S rRNA gene.

The complete double-stranded nucleotide sequence of the cloned *M. flocculare Xba* I fragment is shown in Fig. 3.15. The 665 bp sequence was compared with the *M. hyopneumoniae* 5S rRNA sequences and a 106 bp sequence of the cloned fragment was found to have 95.3% homology with the *M. hyopneumoniae* 5S rRNA gene. A comparison of the nucleotide sequence of the *M. flocculare* 5S rRNA gene and its flanking regions with that of the *M. hyopneumoniae* 5S rRNA gene and its flanking regions is shown in Fig. 3.16. The five different nucleotides in sequences of the *M. flocculare Xba* I fragment and the *M. hyopneumoniae* 5S rRNA gene were confirmed by double strand sequencing. A weaker band was found when the *M. hyopneumoniae* 5S rRNA specific oligonucleotide probe, Mhp5S17mer, was used to hybridize with *M. flocculare* genomic DNA comparing to the result from the hybridization experiment when *M. hyopneumoniae* genomic DNA was used. These data further support the two nucleotide difference (TA vs AT) between the reported *M. hyopneumoniae* 5S rRNA gene sequence and our *M. flocculare* 5S rRNA gene sequence. The homology between the two organisms outside the 5S rRNA coding region was very low. At the -10 regions of the 5S rRNA genes, both *M. flocculare* and *M. hyopneumoniae* had the highly conserved Pribnow box sequences, TATAATA, which had been found in promoters from many other prokaryotic genes (Rosenberg and Court, 1979; Lindahl and Zengel, 1986; Glaser *et al.*, 1992). At the -35 regions of the 5S rRNA genes, no conserved hexanucleotide was present in either organism. Instead, *M. flocculare* had a stretch of 18 "T" residues at this region while *M. hyopneumoniae* had a stretch of 36 "A" residues. *M. hyopneumoniae* had a stretch of 22 "T" residues at the 3' end of the 5S rRNA genes and *M. flocculare* had an "A" rich region there. At the 3' end of the *M. flocculare* 5S rRNA gene, a possible stem loop structure (Fig. 3.17) was found which might serve as terminator. Δ G was obtained using PCGENE (Intellgenetics, Inc., Mountain View, CA, USA).

The results from primer extension experiments showed a major band with a size corresponding to the start point of mature *M. flocculare* 5S rRNA . No evidence of any processing of pre-5S rRNA was found.

Fig. 3.15. Complete double-stranded nucleotide sequence of the cloned *M. flocculare Xba* I fragment, from 5' terminus to 3' terminus and from left end to right end. The 5S rRNA sequences are underlined. "5'>" and "<3'" indicate the 5' and 3' ends of the 5S rRNA sequences.

```
             10         20          30          40

1   AAAATTTAAT TTTCTTGTTT TTCTTGTAAT TATAGCTAAA AAATGCGGTT  50
    TTTTAAATTA AAAGAACAAA AAGAACATTA ATATCGATTT TTTACGCCAA

51  ATTTTGAGCA TAAAATATAG GAAAAGCCAA TTTTTGGGAC AAAATTATGC  100
    TAAAACTCGT ATTTTATATC CTTTTCGGTT AAAAACCCTG TTTTAATACG

101 TAAAAAAAGT TGTTAAAATA GTTGCAAAAA CTTTTTTTTT TTTTTTTTTA  150
    ATTTTTTTCA ACAATTTTAT CAACGTTTTT GAAAAAAAAA AAAAAAAAAT
                                          5'>
151 CATTTTCTAT AATATTTATG CTATAATATT GTTCACAAGA GTAACAATAG  200
    GTAAAAGATA TTATAAATAC GATATTATAA CAAGTGTTCT CATTGTTATC

201 CAAAAGGGAT CACCTGATAC CATTCCGAAC TCAGTAGTTA AGCCTTTTTA  250
    GTTTTCCCTA GTGGACTATG GTAAGGCTTG AGTCATCAAT TCGGAAAAAT
                                               <3'
251 CGCCGAAAAT ACTAGAAATA GGAAAATAGG AAGTTACTCT TTTTTTAGTT  300
    GCGGCTTTTA TGATCTTTAT CCTTTTATCC TTCAATGAGA AAAAAATCAA

301 TTCTTGAAAA TGTGTAAAAA AATATACATT TTAGCAAATA GTTCTACACT  350
    AAGAACTTTT ACACATTTTT TTATATGTAA AATCGTTTAT CAAGATGTGA

351 TTTAAAAAGT ATCGTAATTT ATAGTACTTT TTAAAATAAG ACTCGGTAAA  400
    AAATTTTTCA TAGCATTAAA TATCATGAAA AATTTTATTC TGAGCCATTT

401 ACAAAACGCC GGAAAAGAGG GGAAAGATAT GGAAAATTTA CTTAAAAAAT  450
    TGTTTTGCGG CCTTTTCTCC CCTTTCTATA CCTTTTAAAT GAATTTTTTA

451 TTACAAAGCG TTCCTTTTTG CAGCGCTTTC GGCCTTGCAA TAATTTTCTC  500
    AATGTTTCGC AAGGAAAAAC GTCGCGAAAG CCGGAACGTT ATTAAAAGAG

501 TAGTAATGAG ATTCTTCAAA ATGAGGAGAC TGTTATTAAA TAAAAACAGC  550
    ATCATTACTC TAAGAAGTTT TACTCCTCTG ACAATAATTT ATTTTTGTCG

551 AGAAAATTGA AATAAAAAGT CGTAAAATTC ATATAAAATC AGCCAAGAGT  600
    TCTTTTAACT TTATTTTTCA GCATTTTAAG TATATTTTAG TCGGTTCTCA

601 TACCTCAGTT TCTAATCATA TGCTAATAAA ATTCCAAAAT CTGTTCTAGA  650
    ATGGAGTCAA AGATTAGTAT ACGATTATTT TAAGGTTTTA GACAAGATCT

651 GACGCTGCAG GCAGT
    CTGCGACGTC CGTCA
```

Fig. 3.16. Nucleotide sequence of the *M. flocculare* 5S rRNA gene and the flanking regions and sequence alignment with that of *M. hyopneumoniae* 5S rRNA gene.

```
                                                                   -10

Mf.5S:     AACTTTTTTTTTTTTTTTTTTACATTTTCTATAATATT-TATGCTATAATA
                                         Pribnow box      Pribnow box
Mhp.5S:    **AAAAAAAAAAAAAAAAAAA*A*AAAAAA*A**A*AAA****GTATAATA
                                                          Pribnow box
                 5'>       10        20        30        40

Mf.5S:     TTGTTCACAAGAGTAACAATAGCAAAAGGATCACCTGATACCATTCCGAA
                                         5S rRNA
Mhp.5S:    **AA*T*T******************************AT**********

               50        60        70        80        90

Mf.5S:     CTCAGTAGTTAAGCCTTTTTACGCCGAAAATACTAGAAATAGGAAAATAGG

Mhp.5S:    ****************************G**********************

              100  <3'

Mf.5S:     AAGTTACTCTTTTTTTAGTTTTCTTGAAAATGTGTAAAAAAA

Mhp.5S:    G***************TT****T**TTTTT*A*A**T*T***
```

Mhp., *M. hyopneumoniae*; Mf., *M. flocculare*.

* Indicates a nucleotide that was identical to the *M. flocculare* nucleotide.

Fig. 3.17. Putative terminator structure at the 3' end of the *M. flocculare* (Mf) 5S rRNA gene.

```
                                                                AAUUU
                                                               U     A
                                                               G     U
                                                                C    A
                                                                 U  G
                                                                  A*U
                                                                  U*A
                                                                  G*C
                                                                  A*U
                                                                  A*U
                                                                  A*U
                                                                  A*U
                                                                  A*U
                                                                  U*A
                                                                  U*A
      <3'                                                         U*A
Mf5S-UUUUUUUAGUUUUCUUGAAAAUGUGUAAAAAAAAUAUACAUUUUAGCAAAUAGUUCUACACU*AUAAGACUCGGUAAAACA
```

Δ G (25°C)= -8.4 Kcal

CHAPTER IV. DISCUSSION

4.1 Comparison of the restriction endonuclease cleavage patterns of the chromosomal DNA from *M. hyopneumoniae* and *M. flocculare* and their rRNA gene hybridization patterns.

M. hyopneumoniae and *M. flocculare* are two closely related porcine mycoplasmas. It is technically demanding to distinguish these two species by conventional identification techniques. Razin and several other researchers have proposed (Bove and Saillard, 1976; Chan and Ross, 1984; Razin, 1985, 1992) that the cleavage patterns produced by electrophoresis can be considered genomic fingerprints and comparison of their DNA chromosomal cleavage patterns and their rRNA gene hybridization patterns might provide a way to distinguish these two organisms and to study the genetic relatedness between them or with other mycoplasma species. In this study, the restriction cleavage patterns of two porcine mycoplasmas, *M. hyopneumoniae* and *M. flocculare*, were compared (Fig. 3.1).

Since the mycoplasmal DNA has typically DNA with a low mol % G+C content (Razin, 1985), restriction enzymes with recognition sequences rich in A+T residues were applied to produce large number of small DNA fragments. The distribution patterns of the sizes of the restriction fragments generated by digestions of genomic DNA from the two mycoplasma species with these enzymes were different with many distinguishable bands (Fig. 3.1). However, these distribution patterns provided very little information about the genetic relatedness between tested mycoplasma species. The main reason to choose these enzymes was to study the organization of the rRNA genes. These enzymes were more likely to have recognition sites in the spacer regions between the rRNA genes as well as in the coding regions of the rRNA genes. Analysis of the number of the bands reacting with each of the three rRNA probes (16S, 23S, 5S) and comparison of the hybridization patterns generated by these enzymes could provide useful information about the number of each mycoplasmal rRNA gene and the position relative to each other. In this study, several different rRNA probes were used to hybridize with restriction enzyme digested mycoplasma genomic DNA (Table 3.2). At the early stage, partially purified *E. coli* rRNA (16S and 23S) was used. Sometimes, these partially purified rRNA probes reacted with both the DNA fragment containing the 16S rRNA gene and the fragment containing the 23S rRNA gene with different intensity; it was thus decided that only strong bands should be used to interpret the results. By combining the hybridization data from all the enzymes tested, it was made clear that the *M. flocculare* 16S and 23S rRNA genes were closely linked. In no case did more than one fragment react with all three rRNA probes (16S, 23S, and 5S) which ruled out the possibility of existence of multiple copies of the rRNA genes (Table 3.2). When the cloned rRNA probes were later available, the hybridization data confirmed these early findings (Table 3.5). Because the results from these partially purified rRNA probes have provided adequate information about number and order of the mycoplasma rRNA genes, it was decided that it was not necessary to repeat the early parts of the hybridization experiments using the purified rRNA probes. The hybridization data of the rRNA genes show that *M. flocculare*, like *M. hyopneumoniae*, had a single copy of each rRNA gene (16S, 23S, 5S) and its 5S rRNA gene was distant from the closely linked 5'-16S-23S-3' rRNA cluster (Table 3.2). The rRNA genes and their products are highly conserved throughout prokaryotic organisms, including the mollicutes (Glaser *et al* 1992; Razin 1985). The number of rRNA gene operons and the order of the rRNA genes could be used as more effective phylogenetic markers to study the genetic relatedness among mollicutes. The resemblance of the number and order of the rRNA genes, especially the orphan 5S rRNA genes, gives further support to the concept that these two porcine mycoplasma species are more closely related to one another than to other mycoplasma species and they might form a separated taxonomic group.

The restriction endonucleases with recognition sequences rich in G+C residues cut the intact mycoplasma chromosomes at fewer sites and produced a smaller number of large fragments. By using field-inversion gel electrophoresis, it was possible to achieve high resolution size separation of these large DNA fragments and to compare the cleavage patterns of the two organisms with the enzymes (p. 54). The DNA cleavage patterns of these enzymes were easier to compare on the field-inversion electrophoresis gels than on conventional gel electrophoresis. The cleavage patterns of these two porcine mycoplasmas were reproducible, characteristic of the mycoplasma species tested, and different (Fig. 3.2, 3.3). Each enzyme tested digested the intact genomic DNA from *M. hyopneumoniae* or *M. flocculare* into a different number of fragments with different sizes. The analysis of the restriction patterns of these enzymes indicated that these two porcine mycoplasma species were quite different genetically, even though they are considered more closely related to each other than to other mycoplasma species. Clearly the genetic relatedness between randomly selected mycoplasma species based solely on restriction pattern data has no phylogenetic or taxonomic value. The results obtained in this study seem to indicate that, like other enzymes (p. 52), the enzymes recognize restriction sites rich in C+G (p.54) can provide limited information about genetic relatedness among mycoplasma species. However, these results might be useful for determining genetic homogeneity or heterogeneity of strains within established mycoplasma species (Razin, 1989, 1992; Razin *et al.*, 1983b, 1983c). Frey *et al.* (1992) reported markedly different restriction patterns obtained from the chromosomes of phenotypically similar *M. hyopneumoniae* strains. It was noted that these *M. hyopneumoniae* strains could be put into two groups based upon *Apa* I restriction patterns. Restriction patterns were one of many criteria used in the separation of the *U. urealyticum* serotypes into two genotypic clusters as well (Harasawa *et al.*, 1990). When the sequences of the *M. hyopneumoniae* and *M. flocculare* 16S (Stemke *et al.*, 1992) and 5S (this study) rRNA genes were compared, they showed more than 95% homology (Fig. 3.16). It provides firm evidence of the close phylogenetic relationship between these organisms. However, the sequences of the flanking regions of these two species differed greatly (Fig. 3.16). Since mycoplasmas have high mutation rates and are in a state of rapid evolution (Woese *et al.*, 1980, 1985), the mutations in the functional units tend to be lethal and thus lost, while mutations in non-functional regions are more likely be neutral and thus remain. Razin suggested (1985) that comparison of hybridization patterns of the mycoplasma genomic DNA with conserved gene probes, such as rRNA gene probes, might provide more reliable information about genetic relatedness among mycoplasma species than comparison of the whole genomic DNA cleavage patterns. Hybridization data, using defined rRNA probes to restriction enzyme-digested mycoplasmal genomic DNA, showed different patterns between two closely related mycoplasma species, *M. hyopneumoniae* and *M. flocculare*. For the closely related mycoplasma species, the restriction sites within the rRNA coding regions are more likely to be conserved so these genes will tend to be cut into the same number of fragments and thus they should have the same number of hybridization bands. On the other hand, the changes at the restriction recognition sites outside the rRNA coding regions could change the sizes of the hybridization bands accordingly.

In conclusion, my data indicated that the digestion patterns of restriction enzymes, including enzymes with recognition sites rich in A+T content or rich in G+C content, did not provide much information about phylogenetic relationship among mycoplasma species. Analysis of hybridization patterns of the highly conserved rRNA genes was not helpful in this sense either. However, the number of the rRNA gene operons and the order of the rRNA genes can be used as more effective phylogenetic markers to study the genetic relatedness among mycoplasma species while restriction patterns of the restriction enzymes with recognition sites rich in G+C might be used to study the phylogenetical relationship among the different isolates and strains of the same mycoplasma species.

4.2. Comparison of the restriction endonuclease patterns and the rRNA gene hybridization patterns of *M. hyorhinis* with other two porcine mycoplasmas.

Restriction patterns and rRNA gene hybridization patterns of *M. hyorhinis* were compared with that of *M. hyopneumoniae* and *M. flocculare*. Previous studies have shown weak antigenic cross-reactivity between *M. hyorhinis* and *M. hyopneumoniae*. DNA/DNA hybridization between them was about 5% (Razin, 1985; Stemke *et al.*, 1985). For the enzymes tested (p.58), no major difference could be detected between the restriction patterns of *M. hyorhinis* and those of *M. hyopneumoniae* and *M. flocculare*. It is difficult to study the phylogenetic relationship among these three porcine mycoplasmas based upon the restriction patterns of these enzymes even though two of them, *M. hyopneumoniae* and *M. flocculare*, have been proven to be more closely related to each other than to *M. hyorhinis* (Stemke *et al.*, 1992). The restriction patterns of other enzymes (p. 59) were quite different between *M. hyorhinis* and the other two organisms. For example, digestion of *M. hyopneumoniae* and *M. flocculare* DNA with *Sal* I generated more than ten fragments while digestion of *M. hyorhinis* DNA with *Sal* I only produced two small fragments (210 and 120 kbp, see Table 3.4) and a large fragment, which stayed at the top of the lane because the fragment was too large to move into the FIGE gels under the experimental conditions used in this study. A similar situation was found in the cases of *Mlu* I and *Bgl* I digests as well (Table 3.4). Many of these enzymes (p. 59), which can digest the intact *M. hyopneumoniae* and *M. flocculare* genomes to completion, showed no or partial digestion with *M. hyorhinis* DNA. The genome of *M. hyorhinis* reportedly has a high frequency of methylated bases, especially m^{5}Cyt (Razin and Razin, 1980). Methylated bases in the enzyme recognition sites could make the DNA resistant to digestion. The data obtained from this study (Table 3.1, section 3.2) showed that *M. hyorhinis* DNA was resistant to digestion by enzymes with recognition sequences rich in G+C residues, but not enzymes with recognition sequences rich in A+T residues. Base methylation might be the reason for this resistance. Further work is required to substantiate this assumption. Another reason could be the present of inhibitors which were introduced during the DNA preparation. Several different DNA preparations, different DNA concentrations, and a pair of enzymes that recognize the same site (*Sno* I/*Apa* L I) were tried and gave the same results. These results indicated that inhibition may not be the principal cause of the resistance of *M. hyorhinis* DNA to enzyme digestion. It was suggested (Razin, 1985, 1992) that the methylation sequence specificity could be a useful criterion for differentiating mycoplasmas, because it reflected the nucleotide sequence recognized by the DNA methylase(s) of the organism. The results obtained in this study gave some support to this concept and added to the conclusion that, within this taxonomic group, *M. hyopneumoniae* and *M. flocculare* were closer to each other than to *M. hyorhinis*.

Hybridization results using rRNA probes to restriction enzyme digested *M. hyorhinis* DNA are shown in Table 3.2. The hybridization patterns did not reveal any significant difference between *M. hyorhinis* and the other two species. These results do not support the suggestion (Razin, 1985, 1992) that the hybridization patterns of the conserved genes could provide useful information about phylogenetic relationship among mycoplasma species. These data also showed that *M. hyorhinis*, like *M. hyopneumoniae* and *M. flocculare*, had a single set of each rRNA gene (16S, 23S, 5S) and its 5S rRNA gene might be separated from the closely linked 16S-23S rRNA genes (Table 3.2). An orphan 5S rRNA gene in all three porcine mycoplasmas seems to be a important feature which could help distinguish them from other mycoplasma species. Comparison of 16S rRNA sequences also indicated the close genetic relationship among these three mycoplasma species. Based upon these results, it was shown (Stemke *et al.* 1992) that these three porcine respiratory mycoplasma species form a taxonomic cluster among the mollicutes. It will also be interesting to look at other porcine mycoplasmas.

4.3. Physical maps of *M. hyopneumoniae* and *M. flocculare* and the localization of the rRNA genes on these maps.

The hybridization results of the digested genomic DNA with the rRNA probes from this study confirmed that *M. flocculare*, like *M. hyopneumoniae*, had one copy of each rRNA genes and the 5S rRNA gene was separated from the closely linked 16S and 23S rRNA genes (Table 3.5). The comparison of the physical maps, and eventually the genetic maps, of the two organisms could provide more information about their genomic DNA structure and their phylogenetic relationship with other mollicutes.

In this study, the restriction maps for *M. flocculare* ATCC 27716 and *M. hyopneumoniae* ATCC 25095 have been constructed (Fig. 3.5). The genome size of *M. flocculare* is 890 kbp and that of *M. hyopneumoniae* is 1070 kbp (Robertson *et al.*, 1990). Therefore, restriction enzymes with 10 restriction sites on the genomes will give a mean resolution of 90 kbp for *M. flocculare* and 110 kbp for *M. hyopneumoniae*. When more than two different such enzymes are applied together, a physical map with a mean resolution of less than 50 kbp could be constructed. The complete restriction maps of two enzymes (*Apa* I and *Sno* I) for *M. hyopneumoniae* and that of three enzymes (*Asp*718 I, *Apa* I, and *Sal* I) for *M. flocculare* are shown in Fig.3.5a and 3.5b. The validity of these maps depends upon the accuracy and soundness of the data used in its construction. In all cases, this is a question of the accuracy of the determination of sizes of the DNA fragments generated by the restriction enzymes.

DNA mobility during field-inversion gel electrophoresis (FIGE) was sensitive to a variety of molecular properties, which can produce anomalous electrophoretic behavior for some DNA under different conditions and introduce uncertainty into DNA size determinations (Cantor *et al.*, 1988; Carle *et al.*, 1986; Ellis *et al.*, 1987). In this study, it was found that under the experimental conditions applied, the large DNA fragments (>350 kbp) migrate faster than the smaller DNA fragments on the FIGE gels

(Fig. 3.2). For example, the size of *Asp* f1 was 270 kbp according to its position on the FIGE gels with respect to the position of the molecular size markers. However, by summing the double digestion fragments of *Asp* f1, it was shown that its actual size was 380 kbp and this result was confirmed by the data from CHEF. Such "non-monotonic migration" has been reported by Lalande *et al.* (1988) and is a function of pulsed field conditions. The smaller DNA fragments (<300 kbp) migrated basically according to their sizes which exhibited good agreement with the double digestion and CHEF data (Table 3.3). Maniloff (1989) suggested that low G+C mycoplasma DNA exhibited anomalous behavior in FIGE, leading to slower than expected mobility and incorrect genome size values. His argument, while hypothetically valid, was refuted in these specific cases by experiments showing that there is no correlation between genome size and G+C content in mollicutes (Neimark and Lange, 1990; Robertson *et al.*, 1990).

In some cases, the sizes of two DNA fragments were very close to each other and they formed a single strong band on the gel. It was difficult to separate them by changing FIGE experiment conditions. Double digestion of these double bands can be used to analyze these DNA fragments. For example, *Sno* h1 was a double DNA fragments band (Fig. 3.3) which could be digested by *Apa* I to generate four DNA fragments with the sizes of 150 kbp, 140 kbp, 34 kbp, and 44 kbp. Combined with the hybridization of these bands with radioactive isotope labeled specific DNA fragment probes from *M. hyopneumoniae*, these DNA fragments could be identified and their positions could be located on the physical map.

The DNA fragments from *M. hyopneumoniae* or *M. flocculare* were isolated from the agarose blocks and radiolabeled. They were then used as probes to identify the overlapping parts of other DNA fragments in order to locate their position on the physical maps. In some cases, the probe reacted with several DNA fragments with different intensity which made it difficult to interpret these results. Some of these phenomena might be caused by non-specific reaction between the radioactive labeled intragenic region of the probes and intragenic regions of the target DNA due to the high A+T contents in these regions. They could also be caused by repetitive DNA sequences in the genomes of these mycoplasma species. Under high stringency conditions, most of these non-specific bindings could be avoided. Technical difficulties could also be the cause of this problem. For example, it was very difficult to separate two closely located bands on the gels so that these DNA fragments would not be cross-contaminated. To avoid this problem, well separated bands were chosen as probes. When it was impossible to find well separated bands, several different DNA fragment preparation from different gels would be tried. In addition, using strong hybridization bands to locate the large overlapping regions and combined with the double digestion data, it was possible to solve most of the problems.

Success in ordering the DNA restriction fragments depends on combination of double digestion data and hybridization data from the proper overlapping DNA fragment used as probes. Sometimes, it was difficult to find proper overlapping probes for small DNA fragments. For example, the relative position of four small fragments: *Asp* f5/ *Asp* f6 and *Apa* h6/ *Apa* h7, could not be determined due to the lack of proper probes even though double digestion data suggested that they should be arranged in the order shown in Fig. 3.5a and 3.5b.

A physical map of a bacterial genome is presented in most cases as a map of restriction endonuclease cleavage sites or, in rare instances, as a collection of overlapping or adjacent clones in plasmids, cosmids, or lambda phages, as is the map of *E. coli* (Kohara *et al.*, 1987). The main purpose for constructing physical maps is to have a basis for establishing genetic maps of the organisms. This is especially true for bacteria like mycoplasmas (Dybvig, 1990), for which the classical tools for mapping genes cannot be applied or are not as well developed as are those for *E. coli* or *Bacillus subtilis*. The goal of a genetic map is to have genes localized on the genome with respect to each other; therefore, the higher the resolution of the physical map, the better will be the genetic map. There are several ways to convert a physical map into a genetic map. The simplest and most frequently used approach is to use the cloned defined bacterial genes of the same or related species as DNA probes to identify and localize the genes by specific cross-hybridization. In this study, the restriction maps were constructed with a mean resolution of less than 50 kbp, a relatively low resolution. However, these maps readily allowed the rough chromosomal position of any gene to be determined by Southern blotting. For example, cloned 16S rRNA genes (pR133, Laigret *et al.* 1990) and 5S rRNA genes (M13 5S, Huang and Stemke, 1992) were used to locate the rRNA genes on the restriction maps constructed in this study. Due to the relatively low resolution of the restriction maps, it was difficult to decide the precise location of some of the rRNA genes. In the case of *M. flocculare* 5S rRNA gene, it was only possible to locate the gene on a large DNA fragment of more than 100 kbp. In both of the porcine mycoplasmas, a distance of more than 150 kbp separates the 16S and 23S rRNA genes from the 5S rRNA gene; thus the transcription of the separated rRNA gene loci must be controlled by different promoters. The uncoupling of the 5S rRNA gene from the 16S and 23S rRNA genes was a significant difference between these two porcine mycoplasma species and other mollicute species so far analyzed (Taschke *et al.*, 1986; Huang and Stemke, 1992). The preliminary data from this study showed that another porcine mycoplasma, *M. hyorhinis*, might also have a separate 5S gene. It will be interesting to sequence the *M. hyorhinis* 5S rRNA gene and its flanking regions and to compare these sequences with those of the *M. hyopneumoniae* and *M. flocculare* 5S rRNA genes. The next logical step of this study would be to put more defined gene markers on the physical maps and construct more detailed genetic maps for these two porcine mycoplasmas. Since the size of *M. flocculare* is 200 kbp smaller than that of *M. hyopneumoniae,* comparison of the genetic maps might reveal the structure difference between their genomes and provide more information about their phylogenetic and pathogenic relationship. In this study, attempts were made to locate several genetic markers from other mycoplasma species on the physical maps of *M. hyopneumoniae* and *M. flocculare*, but without success. The reason was presumably that they were too poorly conserved among different mycoplasma species so that they did not hybridized with the target genes under the experimental conditions employed in this study.

4.4. Comparison of the restriction patterns of "*Mycoplasma fermentans*" strain; Mfer-A, Mfer-B, and *incognitus* strain (Mi).

The study of restriction patterns and rRNA gene organization was extended to *M. fermentans*, a human mycoplasma which was found in AIDS patients. Analysis of the restriction cleavage results of the enzymes (p. 75, line 7) showed similar restriction patterns with some distinguishable bands between Mfer-A and Mi. When other enzymes (p. 75, line 13-14) were applied, the restriction patterns on the FIGE gels were quite different between these two strains (Fig. 3.6, 3.7, 3.8, 3.9). No enzymes tested produced the same or similar restriction patterns for both "*M. fermentans* " strains. The most surprising finding was that the restriction patterns between Mfer-A and Mfer-B were very different. In fact, there was more difference between Mfer-A and Mfer-B than between Mfer-A and Mi. These results suggested that these two batches of cells (Mfer-A and Mfer-B) were different. When different DNA fragments from these three mycoplasmas were used as probes, it was found that no cross-hybridization occurred between Mfer-B DNA and Mi DNA under high stringency condition (results not shown). The Mfer-A DNA fragments, however, cross-hybridized with both Mfer-B DNA and Mi DNA under the same conditions (results not shown). Mi DNA fragments cross-hybridized with Mfer-A DNA fragments as well (results not shown). The homology between genomic DNA of *M. fermentans* (type strain) and *M. fermentans* (*incognitus* strain) was reported to be greater than 95% (Saillard *et al.*, 1990). The results from this study raised the question whether Mfer-B was an authentic *M. fermentans* strain. Dr. J. Robertson (personal communications) has been able to show convincingly that the harvest labeled "Mfer-B" can be regrown to a culture largely consisting of *M. hominis*, but with a small proportion of

M. fermentans; growth conditions of the original culture determine if *M. fermentans* or if *M. hominis* predominates. These results are thus in accord with the results found in my study and it appears That I have constructed a map of an impure *M. hominis* rather than the type strain of *M. fermentans*. These results demonstrate the importance of identification of mollicutes used in molecular biological studies and also indicate the variation which can occur if working with mixed culture.

4.5. Physical map of *M. fermentans* (*incognitus* strain) and localization of the rRNA genes on the map.

The hybridization results of the digested genomic DNA with the rRNA probes from this study indicated that *M. fermentans* (*incognitus* strain) had two copies of each rRNA genes. The 5S rRNA genes might be separated from the closely linked 16S and 23S rRNA genes. To confirm these results, the macrorestriction map of *M. fermentans* (*incognitus* strain) was constructed and the rRNA genes were located on the map (Fig. 3.12).

The total genome size of *M. fermentans* (*incognitus* strain) was determined to be 980 kbp (Table 3.7), which was 180 kbp smaller than the reported genome size of 1160 kbp (Neimark and Lange, 1990) for another strain of *M. fermentans* (G II strain). A similar situation was reported in *Ureaplasma urealyticum* and *M. mycoides subsp. mycoides* (Robertson *et al.*, 1990).

The main purpose of constructing a restriction map of *M. fermentans* (*incognitus* strain) was to locate the rRNA genes on the map and to study the rRNA organization. When the rRNA genes were located on the map, it was clear that *M. fermentans* (*incognitus* strain) had an unusual rRNA gene organization. The 16S and 23S rRNA genes were close to each other and formed two inverted loci with respect to each other while both 5S rRNA genes were separated from the 16S-23S rRNA gene clusters (Fig. 3.12). In addition, the hybridization data (Table 3.9) indicated that only one of the two 16S/23S rRNA intergenic spacer regions of *M. fermentans* (*incognitus* strain) contained a *Bgl* I restriction site (Fig. 3.12). The 16S/23S rRNA intergenic spacer region of another strain of *M. fermentans*, PG18 (Harasawa *et al.* 1992), does not contain the *Bgl* I restriction site. These data suggest the existence of variation in these regions.

It is interesting to note that mycoplasmas is one of the only a few groups in prokaryotes which had this unusual rRNA gene organization. Several archaebacteria were also found having unusual rRNA gene organization (Kjems and Garret, 1985, 1987; Kjems *et al.*, 1987). How did this phenomenon happen during evolution? I propose that this organization could be caused by insertion or deletion of genomic DNA during evolution. It was reported that mycoplasma genomes had a high mutation rate (Razin, 1985). For example, the ancestor of a mycoplasma species presumably contained two or more rRNA gene loci arranged in the order of 5'-16S-23S-5S-3'. During evolution, a DNA fragment containing the 5S rRNA gene from one locus and the 16S-23S rRNA genes from another locus was deleted. This could generate a mycoplasma species containing a 5S rRNA gene which was separated from the closely linked 16S-23S rRNA genes. Alternatively, the ancestor of a mycoplasma species might have one rRNA locus arranged in an order: 5'-16S-23S-5S-3'. During evolution, a DNA fragment could have been inserted in the spacer between the 23S rRNA gene and the 5S rRNA gene. This event could generate a species containing a 5S rRNA gene separated from 16S-23S rRNA gene cluster as well. Determination of genetic markers near the rRNA genes may in the future allow us to determine the probable evolution events.

4.6. The preliminary restriction map of Mfer-B.

The preliminary macrorestriction map of Mfer-B was constructed using the methods described earlier in this chapter (Fig. 3.16). The sizes of the large DNA fragments, *e.g.* *Mlu* fb1, fb2, *Mlu* -*Sal* fb1, fb2 (double digestion), were determined using CHEF gels with a yeast chromosome ladder. The positions of the 16S and 5S rRNA genes were also determined on the map. In this case, preliminary results showed no evidence of separated 5S rRNA genes. Because of the uncertainty about the identity of this organism, it was decided that a detailed restriction map should not be constructed until the identity of this organism could be determined unambiguously. However, it appear likely that I have constructed a genomic map of a strain of *M. hominis*.

4.7. A model of the secondary structure of the *M. flocculare* 5S rRNA.

The known 5S RNA molecules were suggested to be classified into three types: eukaryotic type (120 bp), prokaryotic 120N type (120 bp, Gram-negative), and prokaryotic 116 N type (116 bp, Gram-positive; Hori and Osawa, 1979). The data from mycoplasma species indicated that they had distinctly shorter 5S rRNA, *e.g.* 109 bp for 5S rRNA in *M. capricolum* rrnB (Glaser *et al.*, 1992; Hori and Osawa, 1979). It was found that size of the *M. flocculare* 5S rRNA was about 106 bp which was determined by DNA sequencing of the corresponding cloned 5S rRNA gene (Fig. 3.15, 3.16)). The precise location of 5' and 3' ends of the mature *M. flocculare* 5S rRNA have not been decided independently, therefore the indicated length was only putative. The lengths of these shorter mycoplasma 5S RNA are listed in Table 4.1. The alignment analysis (Fig. 4.1) indicates that the shorter lengths are due to small deletions in the regions of the RNA comprising helix E-E' and the single-stranded loop formed by helix E (Hori and Osawa, 1979; Hori *et al.*, 1981; Rogers *et al.*, 1985; Taschke *et al.*, 1986; Walker *et al.*, 1982). This block of deletions result in a truncated structure of helix E. The mycoplasma 5S helix E appears to be the only segment of the 5S rRNA partially missing in known functional 5S rRNA. There are no examples of gross deletions in any other parts of the molecule (Delihas *et al.*, 1984).

Delihas and coworkers (1984) suggested that the five 5S rRNA signature positions could be used to distinguish Gram-positive eubacteria from Gram-negative eubacteria. In Gram-positive eubacteria, these were A_{28}, U_{35}, A_{49}, U_{57}, and G_{60}; and for Gram-negative eubacteria, they were C_{28}, A_{35}, U_{49}, G_{57}, and A_{60} (using the numbering scheme in Fig. 4.1) (Delihas *et al.*, 1984; Rogers *et al.*, 1985). Most of the mycoplasma 5S rRNA had Gram-positive signature sequences. In a few cases, 1 or 2 residues had changed, as they had for some Gram-positive eubacteria (Delihas *et al.*, 1984, Rogers *et al.*, 1985). For the porcine mycoplasmas, *M. hyopneumoniae* and *M. flocculare*, the 5S rRNA shared with other mycoplasmas the signatures A_{49}, U_{57}, and G_{60}, but they missed the signature A_{28} and had the Gram-negative signature A_{35} instead of the Gram-positive signature U_{35} (Taschke *et al.*, 1986, This study). The significance of these differences is unknown. However, the 5S rRNA of closely related organisms exhibited homologies in given regions, known as group-specific signatures, and these two positions might be the group-specific signatures for these two porcine mycoplasmas. One could analyze the *M. hyorhinis* 5S rRNA sequences and look for the these signatures. If the same signature A_{35} was found it could further support the conclusion that these three porcine mycoplasmas belong to the same taxonomic group.

The 5S RNA species studied so far assumed basically the same secondary structure (Hori and Osawa, 1979; Delihas *et al.*, 1984). After sequence homology analysis of the *M. flocculare* 5S RNA, determined from the DNA sequences of the cloned *M. flocculare* 5S RNA gene, compared with 5S rRNA sequences of many eubacterial species including mollicutes, a similar secondary structure was found. The structure has also been shared by another porcine mycoplasma, *M. hyopneumoniae*. The model of the secondary structure of the *M. flocculare* 5S RNA is shown in Fig. 4.2. The G+C content (36.7%) of the *M. flocculare* 5S RNA was relatively low compared to the G+C content of the 5S RNA species from other eubacteria, in line with the low G+C content of the *M. flocculare*

Table 4.1 The length of the 5S rRNA from *E. coli*, *B. subtilis*, and mycoplasma species.

Species	Length (bp)	Source or reference
E. coli	120	Brownlee *et al.* 1967
B. subtilis	116	Rogers *et al.* 1985
A. laidlawii	112	Rogers *et al.* 1985
A. modicum	109	Rogers *et al.* 1985
An. bactoclasticum	113	Rogers *et al.* 1985
M. capricolum	108	Hori *et al.* 1981
M. mycoides mycoides	108	Rogers *et al.* 1985
M. mycoides capri	107	Rogers *et al.* 1985
M. pneumoniae	108	Rogers *et al.* 1985
M. gallisepticum	106	Rogers *et al.* 1985
U. urealyticum	104	Rogers *et al.* 1985
M. hyopneumoniae	106	Taschke *et al* 1986
M. flocculare	106	This study

```
                              1         2         3         4         5         6
                              0         0         0         0         0         0
E. coli              UGCCUGGCGGCCGU-AGCGCGGUGGUCCCACCUGACCCCAUGCCGAACUCAGAAGUGAAACGCCGU-A
B. subtilis          --UUUGGUGGCGAU-AGCGAAGAGGUCACACCCGUUCCCAUACCGAACACGGAAGUUAAGCUCUUC-A
L. brevis            --UGUGGUGGCGAU-AGCCUGAAGGAUACACCUGUUCCCAUGCCGAACACAGAAGUUAAGCUUCAG-C
S. faecalis          --UGUGGUGGCGAU-AGCGAGAAGGAUACACCUGUAACCAUGCCGAACACAGAAGUUAAGCUUCUU-A
S. cremoris          -UUUUGGUCAUCAU-UGCGAUGGAGAUACACCUGUUCCCAUGUCGAACACAGAAGUUAAGUCCAUC-U
C. pasteurianum      --UCCAGUGUCUAU-GACUUAGAGGUAACACUCCUUCCCAUUCCGAACAGGCAGGUUAAGCUCUAA-U
C. innocuum          --UCUGGUGGCGAU-AGCGAUGUGGAUACACCUGUUCUCAUCCCGAACACAGAAGUUAAGCAUAUC-A
A. laidlawii         --UCUGGUGACGAUAGGUAAGAUGGUU-CACCUGUUCCCAUCCCGAACACAGAAGUUAAGCAUCUU-A
A. modicum           --K-UGGUGACGAU-GGCGAAGUGGAUCCACCUGUUCCCAUCUCGAACACAGAAGUUAAGCACUUC-A
An. bactoclasticum   --UCUGGUGACGAU-GGCAAAGUGGACACACCUGUUCCCAUCCCGAACACAGAAGUUAAGCACUUU-U
Spiroplasma sp. BC3  --UCUGGUGCUUAU-GGCAUAGUGGACACACCCGUUCCCAUCCCGAACACGGAAGUUAAGCACUAU-U
U. urealyticum       --UGUAGUGAUCAU-AUCAGAGUGGAAAUACCUGUUCCCAUCCCGAACACAGAAGUCAAGCACUCU-A
M. capricolum        --U-UGGUGGUUAU-AGCAUAGAGGUCACACCUGUUCCCAUGCCGAACACAGAAGUUAAGCUCUAU-U
M. mycoides capri    --U-UGGUGGU-AU-AGCAUAGAGGUCACACCUGUUCCCAUGCCGAACACAGAAGUUAAGCUCUAU-U
M. mycoides mycoides --UCUGGUGGU-AU-AGCAUAGAGGUCACACCUGUUCCCAUGCCGAACACAGAAGUUAAGCUCUAU-U
M. pneumoniae        --UUUGGUGCCCAU-GUCGCUGUGGAAACACCUGGUUCCAUUUCGAACCCAGCAGUUAAGCACAGU-G
M. gallisepticum     --UGUGAUAUCGAU-AUCGUGAUGGAAACACCUGGUCCCAUUCCGAACCCAGAAGUUAAGCGUUAUCG
M. hyopneumoniae     -UAAGAGUAACAAU-AGCAAAAGGGAU-CACCUGAAUCCAUUCCGAACUCAGUAGUUAAGCCUUUU-U
M. flocculare        -CAAGAGUAACAAU-AGCAAAAGGGAU-CACCUGAUACCAUUCCGAACUCAGUAGUUAAGCCUUUU-U
                           A            B            C                 C'          B'

                                                    1         1         1
                      7         8         9         0         1         2
                      0         0         0         0         0         0
E. coli              GCGCCGAUGGUAGUGUGGGG-UCUCCCCAUGCGAGAGUAGG-GAACUGCCAGGCAU
B. subtilis          GCGCCGAUGGUAGUCGGGGUUU-CCCCCUGUGAGAGUAGG-ACGCCGCCAAGC--
L. brevis            ACGCCGAUAGUAGUUGGGGAUCGCCCCCUGCGAGGAUAGG-ACGUUGCCACGC--
S. faecalis          GCGCCGAUUGUAGUGAGGGGUUGCCCCUUGUGAGAGUAGG-ACGUCGCCACGC--
S. cremoris          ACGGCGGAAGUACUUGGGGG-UUGCCCCCUGGGAGAUAUGC-GAGUGGCCAAGU--
C. pasteurianum      GUGCUGAUGGUACUGCAGGGGAAGCCCUGUGGAAGAGUAGG-UCGACGCUGGGU--
C. innocuum          GCGGCGACAAUAUCUGGACUG--G-CUCCAGUGAAGAUAGC-ACGCUGCCAGGU--
A. laidlawii         GCGCCGACGAUAGUUA--UUACAA-U--UAGCGAAAAUAGG-ACGUUGCCAGGC--
A. modicum           GCGUCGAAAAUAGUC-C--U--AAG--G-GGCGAAGAUAGA-ACGUUGCCAGGC--
An. bactoclasticum   ACGCUGAUGGUAGU-ACGUU-UAA-UCGU-GCGAGAGUAGG-AAGUUGCCGGGC--
Spiroplasma sp. BC3  ACGCCGACGAUAGC--C---GCAA---G--GUGAAAAUAGG-GCAGUGCCAGGU--
U. urealyticum       GAGCCGAAAAUAGC------GCAA------GU-AAAAUAGG-UCAUCGCUACGC--
M. capricolum        ACGGUGAAGAUAUU-AC--UG--A-U-GU-GAGAAAAUAGC-AAGCUGCCAFUU--
M. mycoides capri    ACGGUGAAAAUAUU-AC-UU---A-U-GU-GAGAAGAUAGC-AAGCUGCCAGUU--
M. mycoides mycoides ACGGUGAAAAUAUU-AC-UU---A-U-GU-GAGAAGAUAGC-AAGCUGCCAGGU--
M. pneumoniae        GAGCCGAAUGUAGCUG--UU-----U--CAGUGAGAAUAGG-AAAGCACCAAGC--
M. gallisepticum     GAGCCAAAGG--UU-GC---GCAA---GCA-A--GAAUAGGAAAAUAUCACGC--
M. hyopneumoniae     ACGCCGAAGAUA-CUA----GAAA----UAG-GAAAAUAGG-GAGUUACUCUUU--
M. flocculare        ACGCCGAAAAUA-CUA----GAAA----UAG-GAAAAUAGG-AAGUUACUCUUU--
                          D        E              E'        D'          A'
```

Fig. 4.1 Comparison of the *M. flocculare* 5S rRNA with 5S rRNA sequences of *Escherichia coli, Bacillus subtilis, Lactobacillus brevis, Streptococcus faecalis, Streptococcus cremoris, Clostridium pasteurianum, Clostridium innocuum, Acholeplasma laidlawii, Acholeplasma modicum, Anaeroplasma bactoclasticum, Spiroplasma sp. BC3, Ureaplsma urealyticum, Mycoplasma capricolum, Mycoplasma mycoides capri, Mycoplsama mycoides mycoides, Mycoplasma pneumoniae, Mycoplsama gallisepticum* (Rogers *et al.* 1985), and *M. hyopneumoniae* (Taschke *et al.* 1986).

```
                            AUCA       ACC
                          G     C    U    A
                UAGCAAAAGG       CUGA      U
              A     ******       ****      U
             A      UUUUCC       GACU      C
5'AAGAGUAAC      U       G     U     C    C
  **********    A         A     A      AAG
3'UUCUCAUUGA   C            AUUG
              A G
              G*C
              G*C
              A G
              U*A
              A A
              A A
              A A
              A*U
              G A
              G*C
              A*U
              U*A
            A    G
              A A
```

Fig. 4.2 A model of the secondary structure of the *M. flocculare* 5S rRNA.

genome. When the secondary structure of the *M. flocculare* 5S RNA was compared to the secondary structures of the prokaryotic 120N type (Gram negative) 5S rRNA (Fig. 4.3) and the prokaryotic 116N type (Gram positive) 5S rRNA (Fig. 4.4), it was noted that the double-stranded stem regions contained compensatory base changes ensuring that the secondary structure is retained despite large differences in the primary sequences. For example, the fourth GC stem pair in the 5'-3'-terminal helix in *E. coli* has changed to an AU pair in *M. flocculare* sequences. The highly conserved prokaryotic 5S RNA sequence CGAAC is found in the *M. flocculare* 5S RNA as well. This sequence was proposed to interact directly with the constant sequence GTΨCG found in tRNA (Forget and Weissman, 1967; Sprinzl *et al.*, 1976) but the experiments where the corresponding sequence in the 5S rRNA gene was deleted clearly disprove such an interaction as an obligate step in translation (Pace *et al.*, 1982).

4.8. The putative transcription regulation mechanism of the *M. flocculare* 5S rRNA gene.

It was reported that *M. hyopneumoniae* had an orphan 5S rRNA gene separated from the closely linked 16S-23S rRNA genes by at least 4 kbp (Taschke *et al.*, 1986) while the *M. gallisepticum* genome contained three widely separated rRNA loci: one contained genes for all three rRNA species and another contained 23S and 5S rRNA genes whereas the third had only a 16S rRNA gene (Chen and Finch, 1989). In this study, it was found that *M. flocculare* also had a separated 5S rRNA gene with a distance of more than 150 kbp from the closely linked 16S-23S rRNA gene loci (Fig. 3.5a). The transcription of these separated rRNA loci could be regulated by their own regulatory elements or they could be cotranscribed with other genes into long precursors and then processed to mature rRNA species.

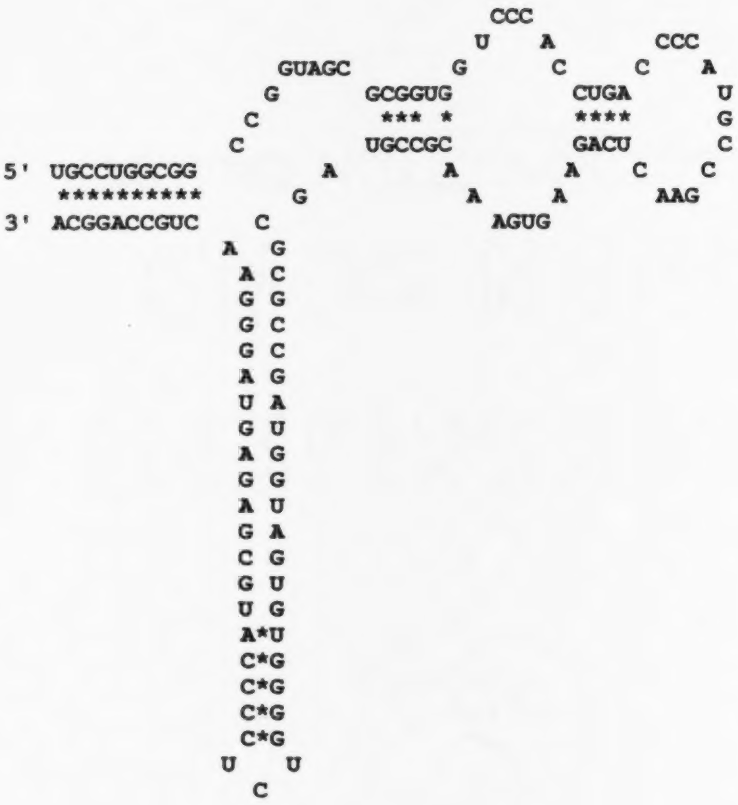

CCC
U A CCC
GUAGC G C C A
G GCGGUG CUGA U
C *** * **** G
C UGCCGC GACU C
5' UGCCUGGCGG A A A C C
********** G A A AAG
3' ACGGACCGUC C AGUG
A G
A C
G G
G C
G C
A G
U A
G U
A G
G G
A U
G A
C G
G U
U G
A*U
C*G
C*G
C*G
C*G
U U
C

Fig. 4.3 A model of the secondary structure of the prokaryotic 120N type (Gram negative) 5S rRNA (Hori and Osawa, 1979).

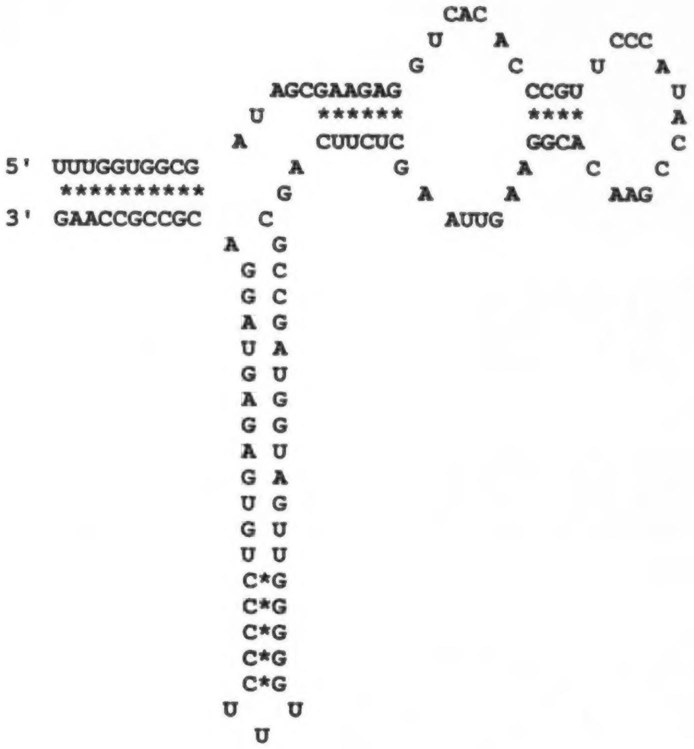

CAC
U A CCC
G C U A
AGCGAAGAG CCGU U
U ****** **** A
A CUUCUC GGCA C
5' UUUGGUGGCG A G A C C
********** G A A AAG
3' GAACCGCCGC C AUUG
A G
G C
G C
A G
U A
G U
A G
G G
A U
G A
U G
G U
U U
C*G
C*G
C*G
C*G
C*G
U U
U

Fig. 4.4. A model of the secondary structure of the prokaryotic 116 N type (Gram positive) 5S rRNA (Hori and Osawa, 1979).

It was found that the *M. flocculare* 5S rRNA gene was located in the middle of the *Xba* I DNA fragment (Fig. 3.15) which I cloned. Sequence analysis of the cloned *Xba* I fragment revealed a long stretch of "T" residues at 5' ends of the *M. flocculare* 5S rRNA gene, while *M. hyopneumoniae* 5S rRNA gene had a long stretch of "A" residues at the 5' end and a stretch of "T" residues at the 3' end (Taschke *et al.*, 1986). The biological function of these sequences is not clear. Studies from several groups showed that many mycoplasma rRNA gene operons were preceded by a consensus Pribnow box at the -10 regions and start transcription with a purine as in other bacteria, while the -35 regions bear no resemblance to the consensus -35 recognition sequences (Brosius *et al.*, 1981, Rosenberg and Court, 1979; Taschke *et al.*, 1986). Instead, the -35 regions are very A+T rich (Hyman *et al.*, 1988; Rasmussen *et al.*, 1987; Taschke and Herrmann, 1986). In this study, the 5S rRNA genes in *M. hyopneumoniae* and *M. flocculare* were preceded by the consensus Pribnow box, TATAATA, at the -10 regions (Fig. 3.16, Taschke *et al.*, 1986). At the -35 region, *M. flocculare* had a long stretch of "T" residues while *M. hyopneumoniae* had a long stretch of "A" residues. Taschke and Herrmann (1986) raised the question of whether the typical -35 consensus could be replaced by an A+T rich region or whether a recognition site was completely dispensable in *M. hyopneumoniae.* At the 3' end of the *M. flocculare* 5S rRNA gene, a stem-loop structure was found (Fig. 3.18). The hall-mark features of DNA sequences of intrinsic terminator are regions of hyphenated dyad symmetry, followed by multiple "T" residues, yielding an RNA with a stem-loop structure followed by a run of "U" residues. Some factor-dependent terminators, however, retain only less stable stem-loop structure (*i.e.* primary A-U pairing rather than G-C pairing) (Broisius *et al.*, 1981; Cheng *et al.*, 1991; Holmes *et al.*, 1983; Rosenberg and Court, 1979). The proposed stem-loop structure found at 3' end of the *M. flocculare* 5S rRNA gene would be apparently a less stable structure because of its primary A-U pairing. These findings could be the further indications that transcription of the orphan 5S rRNA gene in *M. flocculare* was regulated by its own promoter and terminator.

Several different systems are available to study the promoters of the mycoplasma rRNA operons, *e.g. in vitro* transcription, *in vivo* transcription, primer extension, *etc.* Because the enzymes from mycoplasma are not readily accessible, the *E. coli* systems are routinely used as alternatives. The promoter of the single rRNA operon of *M. pneumoniae* functioned *in vitro* using *E. coli* RNA polymerase but no transcripts could be detected *in vivo* (Hyman *et al.*, 1988). In the case of the cloned rrnA operon of *M. capricolum*, the promoters were transcribed by *E. coli* RNA polymerase both *in vitro* and *in vivo* (Gafny *et al.*, 1988). This difference may be due to the presence of the -35 region in the promoter of the rrnA operon of *M. capricolum* resembling that of eubacterial promoters (Glaser *et al.*, 1992). It is apparent that the transcription regulatory elements in mycoplasmas are different from that in *E. coli* and other eubacteria. In this study, primer extension experiments with use of AMV reverse transcriptase were applied to test the predicted promoter for the *M. flocculare* 5S rRNA gene. The results showed no indication of a larger pre-5S rRNA precursor; presumably this result is due to inadequate sensitivity to detect the very small amount of such a precursor rather than the absence of such.. Because no proper systems for studying the promoter activity in mycoplasma are yet available, the putative promoters in *M. flocculare* 5S rRNA gene were not tested for activity.

In conclusion, the results from this study indicate that *M. flocculare*, like *M. hyopneumoniae*, has a single copy of rRNA genes and its 5S rRNA gene is separated from the closely linked 16S-23S rRNA gene cluster. The results also suggest that the 106 bp *M. flocculare* 5S rRNA gene could be regulated by its own promoter and terminator. The data show that *M. fermentans* (*incognitus* strain) has an unusual rRNA gene organization. The genome of *M. fermentans* (*incognitus* strain) contains two copies of rRNA genes and both 5S rRNA genes are separated from closely linked 16S-23S rRNA gene clusters. I suggest that the mycoplasma rRNA gene organization can be used as an useful tool the study the evolution relationship among mycoplasma species.

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